

**MECHANISTIC INSIGHTS INTO THE ACUTE CARDIOVASCULAR
PROTECTION OF ATORVASTATIN**

Thesis submitted by

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DECLARATION

I, Padmanabhan Shakkottai, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

ABSTRACT

Coronary artery disease is by far the single biggest killer in the UK. Atherosclerosis is the underlying cause of coronary artery disease. Hypercholesterolemia is one of the major risk factors in the aetiopathogenesis of atherosclerosis. 3-Hydroxy Methyl Glutaryl CoA reductase (HMG CoA) inhibitors, known as statins, reduce cholesterol levels and offer mortality and morbidity benefit for those with coronary artery disease. The cardiovascular benefits from statins tend to be significant even in individuals with normal cholesterol levels which suggests that there are non-lipid lowering benefits of statins termed “pleiotropic” effects.

Statins attenuate infarct size expansion due to lethal reperfusion injury either prior to ischaemia or at the onset of reperfusion by activating the pro-survival, Reperfusion Injury Salvage Kinase (RISK) pathway. As the duration of treatment acutely at the onset of reperfusion is too short to make any impact on cholesterol levels, it was hypothesized that the protection observed is due to a pleiotropic effect of the statins.

The mechanism by which statins activate the RISK pathway is not entirely clear. Thus experiments with Langendorff perfused isolated rat heart preparation were used to obtain insights into potential mechanisms for statin’s pleiotropic effects in the setting of ischaemia-reperfusion by using Atorvastatin. Infarct size analysis and Western blot analysis were performed to measure cellular injury and protein activation respectively. Atorvastatin was shown to produce a reduction in infarct size when administered acutely during reperfusion at a dose of 50 micromol/l.

Atorvastatin has affinity to the glucocorticoid receptor at levels comparable to potent glucocorticoids. Glucocorticoids have also been shown to activate pro survival kinases. It was thus hypothesized that the acute cardiovascular effects of Atorvastatin may also be mediated by the glucocorticoid receptor, and would be blocked in the presence of the glucocorticoid receptor antagonist, RU486. However studies undertaken demonstrated inconclusive results as RU486 was itself shown to be protective and it would appear that it worked by an alternative mechanism.

Since it is known that pre-ischaemic delivery of Atorvastatin also activates adenosine, an agent known to be cardioprotective. It was hypothesized that Atorvastatin administered during reperfusion may also protect the heart by an adenosine-dependent mechanism. However, despite the infarct sparing action of pre-ischaemic administration of Atorvastatin being confirmed to be dependent on adenosine binding to its receptor, the protection of Atorvastatin administered during reperfusion was shown to be independent of adenosine.

Finally, the acute effects of statins are believed to be due to their action on the HMG CoA reductase enzyme which results in a decrease in mevalonic acid and isoprenyl intermediates. The isoprenyl intermediates such as Rho A, are inhibitors of the various components of the RISK pathway. We were able to demonstrate for the first time that mevalonic acid abrogates the infarct size reduction of Atorvastatin administered during reperfusion. Western blot analysis did not reveal a significant reduction in active Rho A levels, however there was a trend towards a reduction and this reflected in the infarct size studies. Although not conclusive, these results would suggest that the acute cardiovascular effects of Atorvastatin, when given at reperfusion, were due to its pleiotropic effects on Rho A.

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ABBREVIATIONS:

1. AAR: Area at Risk.
2. ADP: Adenine Dinucleotide Phosphate.
3. AMP: Adenine Mononucleotide Phosphate.
4. AMPk: Adenine Mononucleotide Phosphate activated protein kinase.
5. ANT: Adenine Nucleotide translocase.
6. ATP: Adenine Trinucleotide Phosphate.
7. ATV: Atorvastatin.
8. A2A: Adenosine 2A.
9. BCA: Bicinchoninic acid.
10. BOC: British Oxygen Company.
11. BSA: Bovine Serum Albumin.
12. CABG: Coronary Artery Bypass Grafting.
13. CAD: Coronary Artery Disease.
14. CD: Cluster Differentiation.
15. CK: Creatine Kinase.
16. cGMP: cyclic Guanine Mononucleotide Phosphate.
17. CK-MB: Creatine Kinase-MB.
18. COX2: Cyclooxygenase-2.
19. CRP: C-Reactive Protein.
20. DMSO: Dimethyl Sulphoxide.
21. DNA: Deoxy Ribonucleic acid.
22. DTT: Dithiothreitol.
23. eNOS: endothelial Nitric Oxide Synthase.
24. EPO: Erythropoietin.
25. ERK1/2: Extracellular Signal Regulated Kinase 1/2

26. GCR: Glucocorticoid Steroid Receptor.
27. GDP: Guanine Dinucleotide Phosphate.
28. GGPP: Geranyl Geranyl Pyrophosphate.
29. GLUT4: Glucose Transporter 4.
30. GPCR: G Protein Coupled Receptor.
31. GSK: Glycogen Synthetase Kinase.
32. GTP: Guanine Trinucleotide Phosphate.
33. GTPases: Guanine Trinucleotide Phosphatases.
34. HCO_3^- : Bicarbonate.
35. HMG CoA: 3 Hydroxy 3 Methyl Glutaryl Co-enzyme A.
36. HSP 27: Heat Shock Protein 27.
37. HSP 90: Heat shock Protein 90.
38. ICAM: Intercellular Adhesion Molecule.
39. IL6: Interleukin 6.
40. IP: Intraperitoneal.
41. IPC: Ischaemic Preconditioning.
42. iNOS: inducible Nitric Oxide Synthase.
43. kDa: Kilo Dalton..
44. LDL: Low Density Lipoprotein.
45. L-NAME: N(G) – Nitro - L- Arginine Methyl Ester.
46. LVDP: Left Ventricular Developed Pressure.
47. LVEDP: Left Ventricular End Diastolic Pressure.
48. LV: Left Ventricle.
49. MAPK: mitogen Activated Protein kinase.
50. Mev: Mevalonic Acid.
51. MI: Myocardial Infarction.

- 52. Mito k-ATP: Mitochondrial ATP Dependent Potassium Channel.
- 53. MPTP: Mitochondrial permeability Transition Pore.
- 54. mRNA: messenger Ribo Nucleic Acid.
- 55. NADPH: Nicotinamide Adenine Dinucleotide Phosphate Hydrogenase.
- 56. NF- κ B: Nuclear Factor- κ B.
- 57. NO: Nitric Oxide.
- 58. NOS; nitric Oxide Synthase.
- 59. PAGE: Poly Acrylamide Gel Electrophoresis.
- 60. PAF: Platelet Activating Factor.
- 61. P-Akt: Phosphorylated- Akt.
- 62. PCI: Percutaneous Coronary Intervention
- 63. PDK1: Phosphoinositide Dependent Kinase 1.
- 64. PECAM: Platelet/Endothelial Cell adhesion Molecule.
- 64. PGE₂: Prostaglandin E₂
- 65. PGF_{1 α} : Prostaglandin F_{1 α}
- 66. P_i: Phosphate.
- 67. PI3K: Phospho Inositide 3 Phospho kinase.
- 67. PKB: Protein Kinase B.
- 68. PKC: Protein Kinase C.
- 69. PKG: Protein Kinase G.
- 70. PML: Polymorphonuclear Leukocytes.
- 71. PPAR γ : Peroxisome Proliferators Activated Receptor γ .
- 72. PTEN: Phosphatase and Tensin Homolog deleted on chromosome ten.
- 73. RGB: Red-Green-Blue
- 74. RISK: Reperfusion Injury Salvage Kinases.
- 75. ROS: Reactive Oxygen Species.

- 76. ROCK: Rho Associated Kinase.
- 77. RPP: Rate Pressure Product.
- 78. SD: Sprague-Dawley.
- 79. SDS: Sodium Dodecyl Sulphate.
- 80. SEM: Standard Error Mean.
- 81. SR: Sarcoplasmic Reticulum.
- 82. SPT: 8-Sulpho Phenyl Theophylline.
- 83. TEMED: NNN-Tetra Ethyl Ethylenediamine
- 84. TNF- α : Tumour Necrosis Factor α .
- 85. TTC: 2,3,5 Triphenyl Tetrazolium Chloride.
- 87. UK: United Kingdom.
- 88. UKPDS34: United Kingdom Prospective Diabetic Study 34.

1.0 INTRODUCTION

Cardiovascular disease accounts for about 35% of the deaths in UK and of these 50 % of the deaths are as a result of Coronary Artery Disease (CAD)¹. CAD is the most common cause of death in the UK.¹ It is also a significant cause of morbidity and a significant burden to the healthcare system. This has resulted in the drafting of the National Service Framework for Coronary Artery Disease in the UK and in significant research for various treatment modalities.

CAD presents in various clinical settings including acute myocardial infarction, acute coronary syndromes and angina. Early reperfusion is the treatment of choice. However reperfusion strategies such as percutaneous intervention (PCI) and thrombolysis have a thirty day morbidity of 8.5% and 14 % respectively². Part of the reason for this morbidity, despite successful reperfusion, is thought to be due to the existence of lethal ischaemia-reperfusion injury.

The existence of lethal reperfusion injury over and above the injury caused by ischaemia was first demonstrated in dogs by Jennings et al³. This has subsequently been investigated and potential mechanisms have been demonstrated by various groups such as Hearse et al⁴, Fox et.al⁵, Opie et.al⁶ etc. However early controversy stemmed from the possibility that this extension of infarct in the reperfusion phase could be just a continuation of the necrotic process that commenced in ischaemia. This problem is compounded by the inability to accurately track the development of necrosis with time⁷.

There is now convincing evidence to suggest that lethal reperfusion injury does exist, as interventions targeting the reperfusion phase alone have demonstrated a reduction in infarct size both in animals and in humans⁷⁻¹⁰. It is now clear that

these interventions activate the cell's own protective mechanisms which usually involves a signal transduction cascade that ultimately inhibits apoptosis and necrosis^{11,12}.

The last few years have opened up exciting avenues of research and translational work to characterize various pharmacological agents which are able to harness these protective mechanisms. One of the pharmacological ways of harnessing these protective mechanisms has been via the use of the 3-Hydroxy 3-Methyl Glutaryl CoA reductase inhibitors (HMG CoA reductase) i.e., Statins.

Since the 1990's numerous clinical trials with statins have demonstrated that reduction in serum cholesterol, significantly and safely reduces the incidence of coronary heart disease in those individuals with and without clinically apparent coronary artery disease¹³. Statins reversibly inhibit HMG CoA reductase, which is the rate limiting enzyme in the cholesterol biosynthetic pathway. They also increase hepatic clearance of LDL cholesterol¹⁴. This combined effect has been shown to bring about a very effective reduction in serum cholesterol.

However the benefits from statins appear to be greater than what might be expected from lipid lowering alone¹⁴. This would suggest that there are cholesterol independent or pleiotropic effects of these agents¹⁴. While numerous pleiotropic effects have been demonstrated it is still not clear as to which of these effects are responsible for the attenuation of lethal reperfusion injury in the acute setting.

The aim of the studies outlined in this thesis is to analyse the mechanism and to better understand the cardioprotective effects of acute statin therapy. It is hoped that such research will add to the available pool of information which will

subsequently permit a more rational approach to the use of statins as an adjunct in the treatment of Coronary Artery disease.

1.1 CORONARY ARTERY DISEASE

Coronary artery disease is burgeoning into a medical and socio-economic problem. The amount spent on prescriptions for CAD alone is close to £1.3 billion per annum in the UK. The number of cases of PCI is also rising by 5% per year and there has been a 33% rise in the number of CABG between 1993 and 2006 in the UK¹. Thus a concerted effort has come about to tackle this disease by seeking methods for both primary prevention and secondary prevention. A good understanding of the underlying patho-physiology of the disease process would open up avenues for potential therapeutic measures. This area thus provides a fertile ground for translational research.

CAD is a disease spectrum with acute myocardial infarction representing the most severe form of ischaemic injury and angina representing the mildest form of ischaemia. Atherosclerosis is the most frequent underlying cause for coronary artery disease¹⁵. Atherosclerosis is a chronic immunoinflammatory, fibroproliferative disease of large and medium sized arteries fuelled by lipids¹⁵. Myocardial infarction is precipitated by atherosclerotic plaque rupture and endothelial erosion both of which expose the arterial wall to prothrombotic factors¹⁶. This results in sub-total or total occlusion of the artery and precipitates myocardial ischaemia (**Figure1.01**).

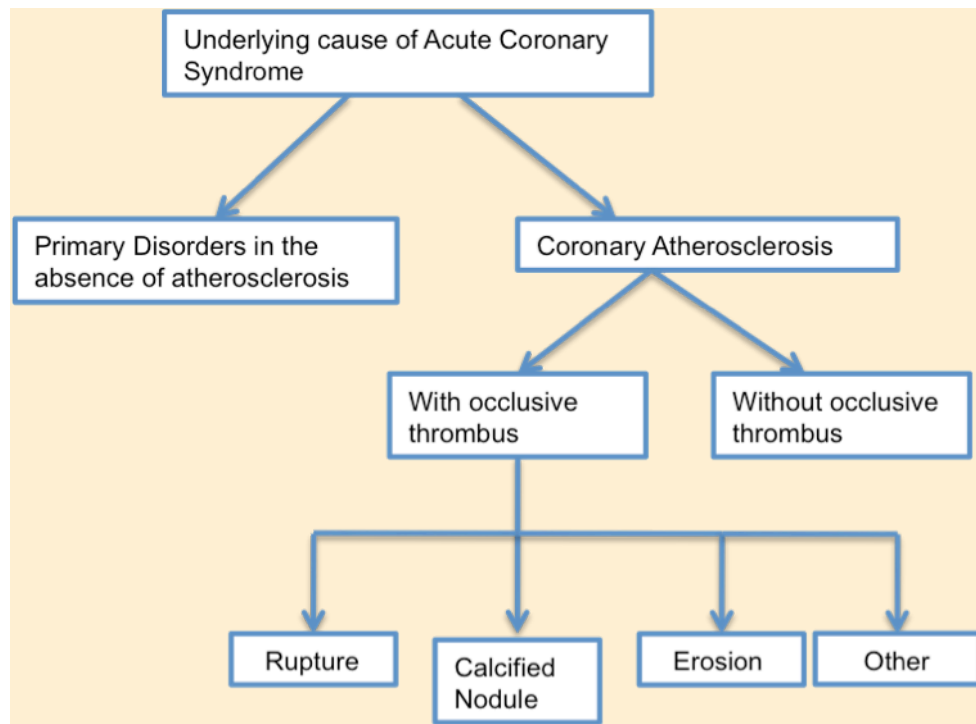


Figure1.01: Various potential mechanisms of atherosclerosis associated coronary artery disease. Plaque rupture is by far the commonest cause of Acute Coronary Syndromes. Adapted and modified from Naghavi et al¹⁷.

Early experiments with coronary artery occlusion in anaesthetized dogs demonstrated the presence of a wave-front of myocardial necrosis spreading from the endocardium to the epicardium and this was shown to be dependent on the duration of ischaemia. Experiments in dogs have demonstrated that the myocardium tends to be reversibly damaged if the period of ischaemia is limited to 15-20 minutes. However if ischaemia persists, myocardial necrosis commences and infarction is complete in 3-6 hours¹⁸. Thus the window for effective myocardial salvage following an infarct is limited and crucial. It is clear that restoration of coronary blood flow to the ischaemic segment is essential as seen in various trials utilizing thrombolytic agents such as GUSTO and GISSI^{19,20}. Percutaneous intervention (PCI) or Coronary Artery Bypass Grafting (CABG) maybe warranted in addition to thrombolysis or even as a primary modality of treatment.

1.1.1 CARDIOPROTECTION AND ISCHAEMIA -REPERFUSION

Despite being essential to prevent further tissue damage, reperfusion strategies such as thrombolysis or PCI are however associated with some infarct extension and this is termed lethal reperfusion injury. The therapeutic window for protection from lethal reperfusion injury could occur during two distinct phases. These are (1) pre-ischaemic phase and (2) reperfusion phase. Pre-ischaemic protective procedures have been associated with the phenomenon of preconditioning. Preconditioning is a very potent cardioprotective mechanism and was discovered over two decades ago. It describes the phenomenon whereby short-lived episodes of myocardial ischaemia and reperfusion, prior to the infarct-inducing episode result in a significant reduction of infarct size²¹. However the clinical potential of this mechanism remains largely unrealised due to the inability to predict the onset of the infarct-inducing episode.

Importantly renewed interest in cardioprotection associated with reperfusion injury was noted when some aspects of classic preconditioning were shown to be dependent on events occurring during the first few minutes of reperfusion^{22,23}. The phenomenon of postconditioning has also contributed to this renewed interest. The term postconditioning has been used to describe the events occurring at reperfusion whereby brief periods of ischaemia interrupting the initial period of reperfusion results in cardioprotection as seen by a reduction in infarct size²³. Various ligands have subsequently been identified which bring about the same effect as mechanical postconditioning and these agents induce what is termed pharmacological postconditioning²⁴. In various animal models and in a human atrial muscle model, postconditioning has been shown to be as effective as preconditioning²⁵.

However despite a thorough understanding of the mechanisms of reperfusion injury, the knowledge of the exact mechanisms by which pharmacological agents intervene in the cascade of reperfusion injury is evolving.

1.2 MYOCARDIAL ISCHAEMIA-REPERFUSION INJURY

In order to gain mechanistic insight into the role of pharmacological agents in the attenuation of lethal reperfusion injury we need to understand the cellular responses to ischaemia and reperfusion. While reperfusion is essential after ischaemia for tissue salvage, it has been noticed that cells which are non-lethally injured during ischaemia can progress towards irreversible cell damage and cell death by either necrosis or apoptosis during early reperfusion⁷.

Reperfusion can result in either a full recovery or precipitate cell death in those cells that are reversibly injured, depending on various factors including the use of cardiovascular protective agents²⁶.

In order to draw a clear line of distinction between myocardial ischaemic injury and lethal reperfusion-injury we need to dissect out the exact sequence of events in each phase.

1.2.1 MYOCARDIAL ISCHAEMIC INJURY

Myocardial ischaemic injury is defined as a state in which arterial blood flow to the myocardium is insufficient to prevent intracellular metabolism from shifting from aerobic respiration to anaerobic metabolism²⁷. However the limited delivery of substrate and the accumulation of metabolites also results in depression in cellular function²⁷. During the first few minutes of ischaemia there is a switch from aerobic metabolism to anaerobic glycolysis. After the first few

minutes the rate of anaerobic glycolysis also declines due to inactivation of glyceraldehyde phosphate dehydrogenase²⁸.

The demand of the ischaemic tissue for high energy phosphate far exceeds the capacity of the reserves and anaerobic glycolysis to provide energy and thus there is a reduction in the intracellular ATP pool. As a result of ATP loss K^+ ions leak out of the myocytes in an effort to retain ionic equilibrium and this leak quickly reaches steady state. However reperfusion at this stage will reverse all these changes.

As a result of anaerobic metabolism the intracellular levels of lactate and metabolites of creatine kinase rise and cause an increase in intracellular osmolarity. This results in water being able to diffuse into the cell but this is partially offset by the exit of K^+ to the extracellular space and thus the cells will not swell. However the cells still retain their structural integrity and reperfusion at this stage is still able to restore homeostasis²⁶.

If ischaemia persists for 30 to 40 minutes then the intracellular adenine nucleotide pool is completely depleted because the cell will utilize all available ATP. ATP is dephosphorylated to ADP and AMP, which in turn are quickly converted to hypoxanthine. As a result protons, phosphate and ammonium ions accumulate resulting in a rise in intra cellular osmolarity. Lactate and other metabolites accumulate and increase the osmolar load which potentiates diffusion of water into the cells. At this stage the cells still retain their structural integrity. However there exists some oedema under the sarcolemmal membrane²⁶.

If ischaemia persists beyond 60 minutes the plasma membrane phospholipids are degraded and lipid peroxidation begins, resulting in loss of cell integrity, cell swelling and eventual cell death (Irreversible cell damage)²⁶. **(Fig1.02)**

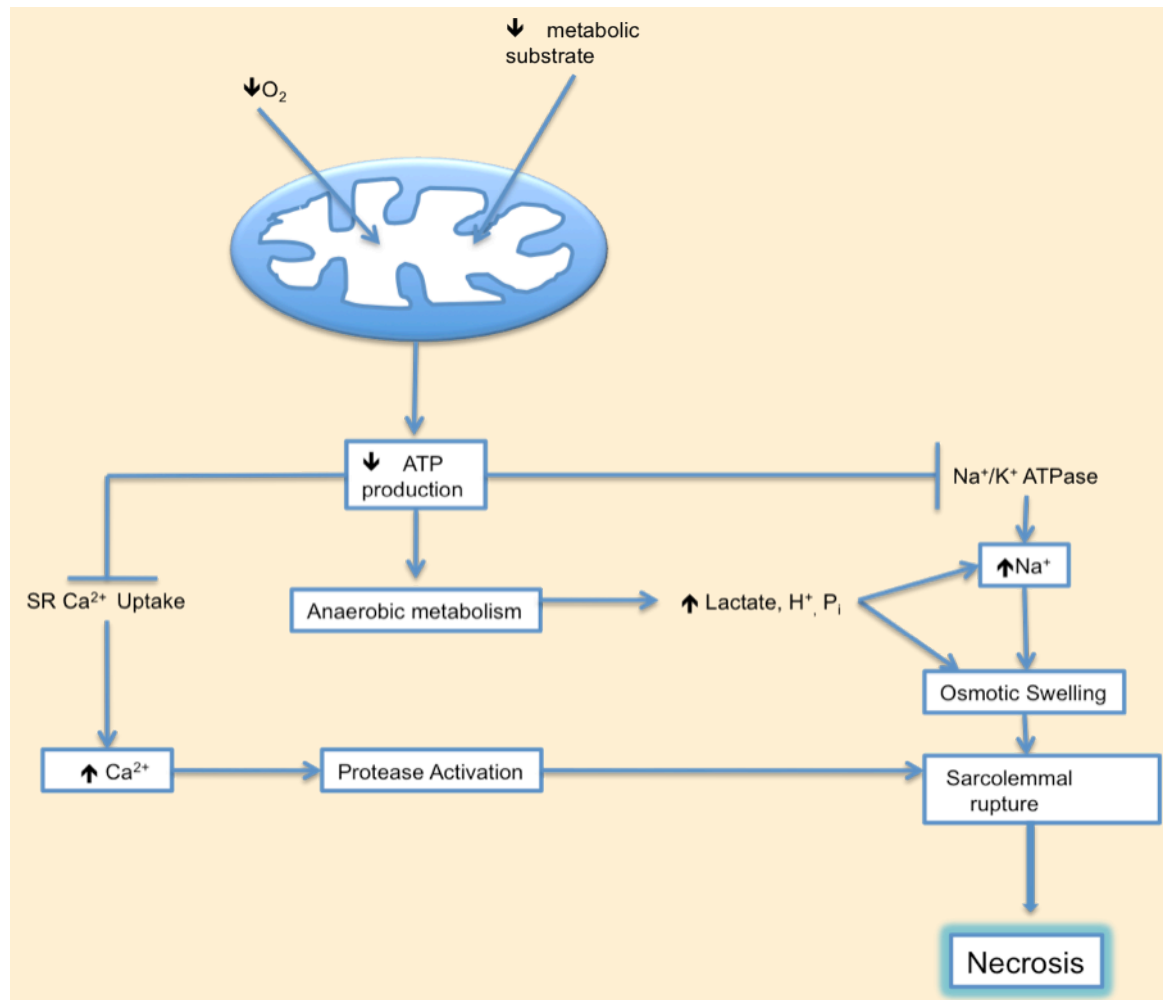


Figure 1.02: The various events occurring during ischaemia result in osmotic swelling, protease activation and eventual sarcolemmal rupture and necrosis. Adapted from Ferdinandy et al²⁹.

1.2.2 ISCHAEMIA REPERFUSION INJURY

Reperfusion injury denotes a causal event associated with reperfusion that has not occurred during the preceding ischaemic period and can be entirely attenuated by interventions delivered at reperfusion⁷. Myocardial reperfusion injury results in four types of injury^{7,30}:

(a) **Myocardial stunning:** This refers to mechanical dysfunction that persists after reperfusion despite restoration of normal or near normal coronary flow³⁰⁻³². This phenomenon is usually responsive to inotropic support in the setting of acute myocardial infarction and the myocardium usually recovers⁷. However this phenomenon is important as a tool for risk stratification of patients with CAD.

(b) **Reperfusion arrhythmias:** These rhythm disturbances are potentially lethal but there are effective treatment strategies available. Reperfusion arrhythmias are defined as disturbances of rhythm that arise as a consequence of a total or partial restoration of blood flow in tissue which has been previously globally or regionally ischaemic³³. These can occur as a result of rapid recovery of action potential characteristics during reperfusion, inhomogeneity of injury resulting in differential return of excitability to tissue and thus supporting re-entry circuits or increased automaticity³³. These reperfusion arrhythmias may also occur due to the presence of free radicals and calcium stress³³. Increased automaticity results in accelerated idioventricular rhythm while re-entry circuits result in ventricular tachycardia and multiple premature ventricular complexes³⁴.

Accelerated idioventricular rhythm is the commonest reperfusion arrhythmia in patients undergoing primary PCI and is associated with extensive myocardial damage and delayed microvascular reperfusion³⁴. However ventricular arrhythmias are also believed to be markers of successful reperfusion in patients with ST elevation myocardial infarction who have been treated with thrombolysis³⁵.

Postconditioning has been effective in abolishing reperfusion arrhythmias in a rat in vivo model of ischaemia-reperfusion and this is related to the opening of

the mitochondrial K_{ATP} channels but is not related to the survival kinases PI3Kinase/AKT³⁶. Conventional anti-arrhythmics such as beta blockers and amiodarone are effective in suppressing these arrhythmias³³. Erythropoietin (EPO) has been shown to abolish ventricular arrhythmias in an in vivo canine model when administered just before reperfusion and this is dependent on PI3 Kinase³⁷. EPO has been shown to abolish premature ventricular complexes and ventricular arrhythmias in murine cardiac myocytes by activation of nitric oxide synthase³⁸. Ranolazine has also been shown to be an effective anti-arrhythmic in a rat in vivo model and this is believed to be due to its inhibition of the late Na^+ current³⁹. Thus effective treatment strategies exist to prevent and control these events.

(c) **No reflow phenomenon:** Loss of microvascular integrity and myocardial oedema results in the absence of subendocardial perfusion despite the presence of normal epicardial flow. This is seen in approximately one third of the patients who have PCI following an acute MI and is defined as poor resolution of ST segment elevation and poor 'myocardial blush' on angiography despite complete recanalization of the infarct-related epicardial artery^{40,41}. This phenomenon is important because it is associated with higher rates of left ventricular dysfunction and mortality; this being partly related to the phenomenon of lethal reperfusion injury which is discussed below.

(d) **Lethal Reperfusion Injury:** This phenomenon is the focus of this thesis. It was first described by Jennings et al in 1960 where they observed cell swelling, myofibril contracture, sarcolemmal disruption and appearance of calcium phosphate crystals in the myocardium during reperfusion⁴². Lethal reperfusion injury is defined as the injury caused by restoration of blood flow following an episode of ischaemia which leads to the death of cardiac myocytes that were

viable at the onset of reperfusion^{7,30,43}. This phenomenon is complex and involves both necrosis and apoptosis which are two distinct mechanisms of cell death. The various alterations associated with lethal reperfusion injury are discussed in the following sections.

1.2.3 LETHAL REPERFUSION INJURY

In order to understand the basis of various cardioprotective strategies a systematic understanding of the various phenomena associated with lethal reperfusion-injury is essential. I will first briefly discuss the main morphological mechanisms of cell death which are necrosis and apoptosis. Cell death is classified according to morphological appearance as apoptosis, necrosis, autophagy and mitosis associated cell death⁴⁴.

1.2.3.1 NECROSIS AND APOPTOSIS

Necrosis: Cell necrosis is generally initiated by non cellular mechanisms such as ischaemia, trauma and thrombosis and results in irreversible cell death characterized by cell swelling, depletion of high energy stores and disruption of cellular membrane due to fluid and electrolyte imbalances^{45,46}. Necrosis usually affects contiguous tract of cells and the release of cellular debris results in inflammation and collateral damage⁴⁷. This process is usually preceded by a 70-100% depletion in ATP levels⁴⁸.

Necrosis is associated with an uncontrolled influx of calcium resulting in mitochondrial calcium overload and ATP depletion⁴⁹. This rapid influx of calcium also inhibits caspase and endonuclease, both of which are involved in apoptosis, and thus preferentially results in necrotic cell death (accidental uncontrolled cell death)⁴⁷. However there now exists evidence to suggest that

necrosis is mediated by signal transduction systems and catabolic mechanisms^{50,51}. This process of calcium influx is overcome by promoting mitochondrial calcium sequestration by the protein bcl2⁵². These mechanisms are described below.

(a) **Default or Fortuitous Necrosis:** Necrosis, as a default pathway for programmed cell death, was first confirmed in baby mice kidney epithelial cells. In this model, exposure to hypoxia both in the in vivo and in vitro setting (when apoptosis and autophagy were inhibited) resulted in necrotic cell death⁵³. In the above model inhibition of apoptosis (and thus mitochondrial membrane permeability) by knock-out of the pro-apoptotic proteins Bax/Bad and inhibition of autophagy by transfection with constitutively active Akt (protein kinase) resulted in necrotic cell death. ATP depletion itself favours the switch from apoptosis to necrosis as sufficient ATP is required for the activation of pro-apoptotic caspases⁴⁸. Nitric oxide can also switch the mode of cell death from apoptosis to necrosis by inhibition of caspases⁵⁴.

(b) **Regulated Necrosis:** Signal transduction has been seen to occur in mice fibrosarcoma cells wherein TNF- α receptor activation is associated with necrosis via activation of death domain⁵¹. This activation is associated with a burst of mitochondrial ROS and increased permeability of outer mitochondrial membrane and is mediated via RIP-1 kinase^{51,55}. This mechanism is known as necroptosis⁵⁶.

(c) **Programmed Necrosis:** Calcium overload and ROS activate cyclophilin D which is associated with the formation of the mitochondrial transition pore and this results in uncoupling of oxidative metabolism and mitochondrial swelling and disruption eventually leading to necrotic cell death⁵⁷.

Necrosis can also be triggered by non-caspase proteases. Calcium overload can trigger the release of proteases such as calpains which act by cleaving proteins in the cytoskeleton and the release of lysosomal enzymes called cathepsins^{58,59}. ROS release results in increased lysosomal membrane permeability and the release of lysosomal enzymes such as cathepsin B & D which eventually results in necrotic cell death⁶⁰.

High levels of reactive oxygen species (ROS) will result in ATP depletion and disruption of intracellular calcium homeostasis and cell death by necrosis⁴⁹. The high levels of ROS also inhibits the apoptotic pathway by direct inhibition of caspases and DNA fragmentation⁶¹.

These various mechanisms are represented in **figure 1.03**.

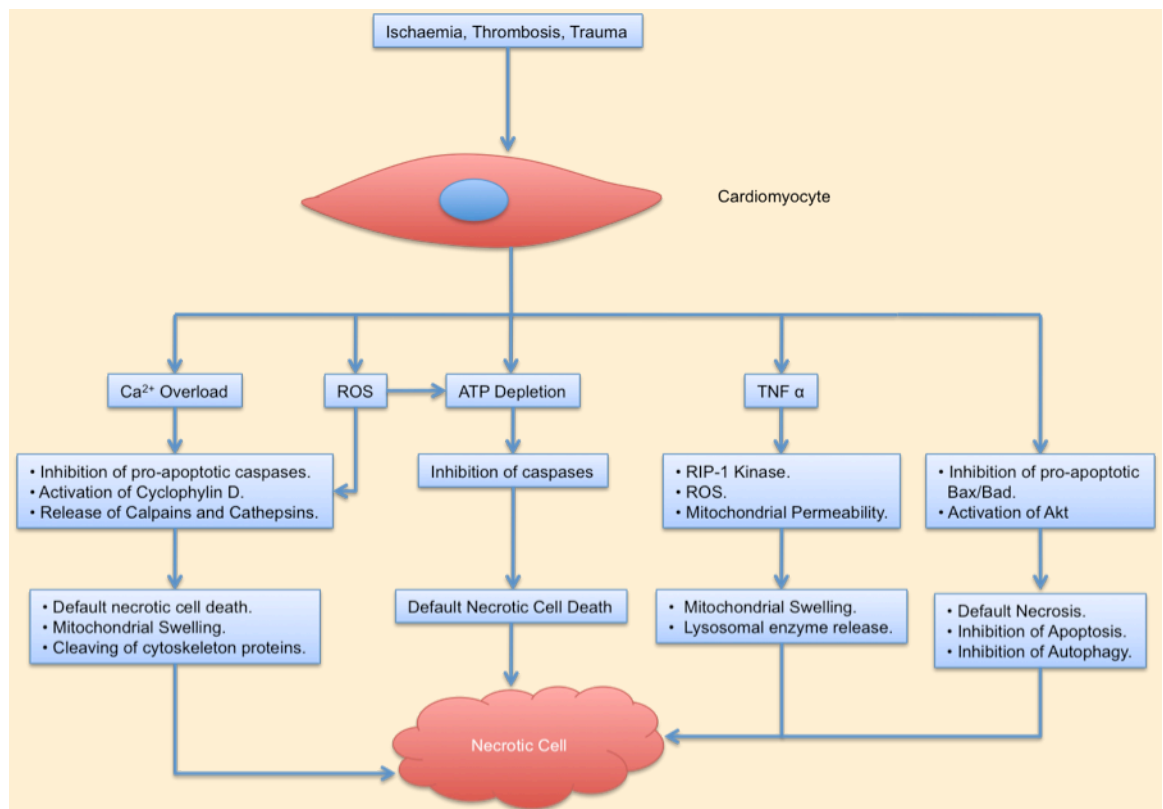


Figure 1.03: Various Mechanisms of Necrosis.

Apoptosis: The term apoptosis was first coined by Kerr et al. to describe a pattern of morphological alterations associated with normal programmed cell death and certain pathological processes in vivo⁶². The changes they described include cell shrinkage, loss of contact with neighbouring cells, formation of cytoplasmic vacuoles, plasma and nuclear membrane blebbing and chromatin condensation.

Apoptosis involves scattered cells within tissues and these are removed by macrophages or adjacent phagocytic cells before the loss of plasma membrane integrity⁶³. It is not associated with inflammatory phenomenon⁶⁴. The differences between necrosis and apoptosis are represented in figures **1.04** and **1.05**.

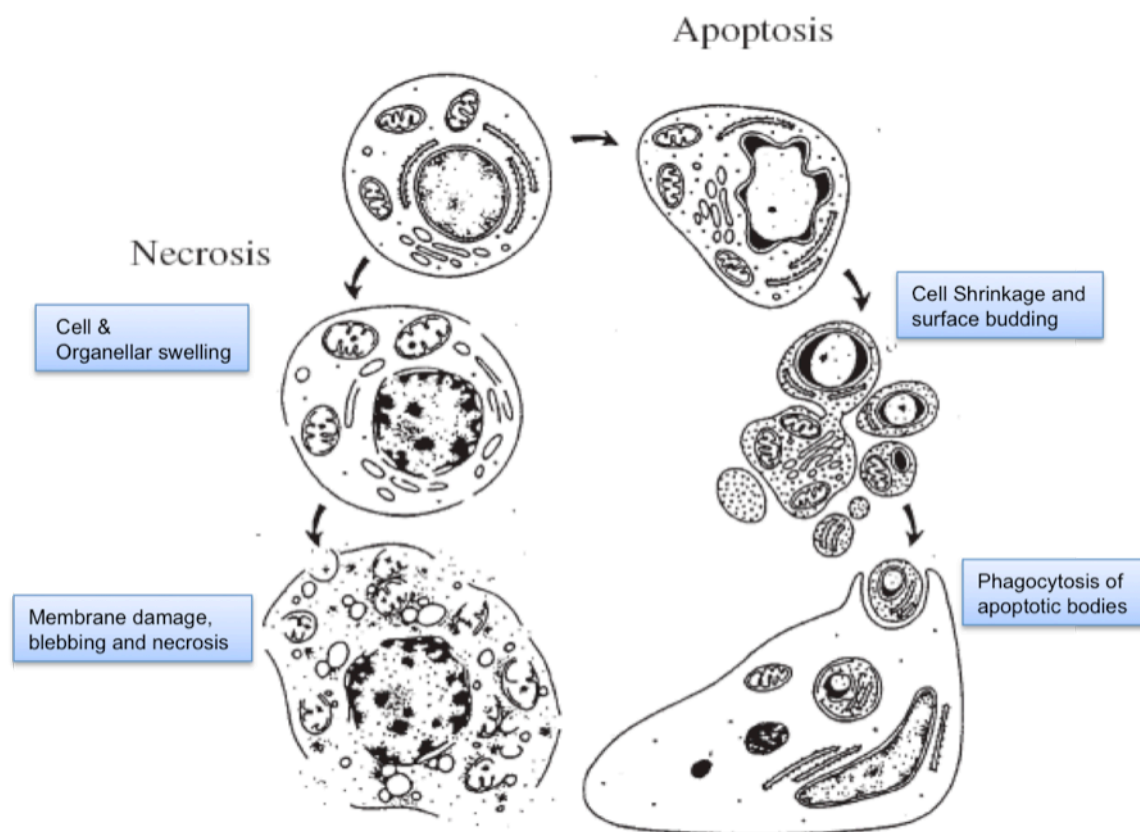


Figure 1.04: Necrosis is characterized by cell swelling, plasma membrane blebbing and cell lysis while apoptosis is characterized by cell shrinkage and phagocytosis. Adapted from Kerr et al⁶².

APOPTOSIS	NECROSIS
(a) Active Process	Passive Process
(b) Cell Shrinkage	Cell Swelling
(c) Intact Membrane	Membrane Rupture
(d) No inflammation	Inflammation
(e) Evolutionarily Conserved pathway.	Toxicant specific biochemical Mechanism.

Figure 1.05: Morphological and functional distinction between Apoptosis and necrosis. Adapted from McConkey et al⁴⁷.

Apoptotic cell death is initiated by the activation of cell surface receptors (extrinsic pathway) or changes in mitochondrial permeability (intrinsic pathway)⁶⁵. The extrinsic pathway involves the direct interaction of polypeptide homologues to the receptor to tumour necrosis factor and CD95/Fas which interact with the 'death pathways' via caspases⁶⁶. The intrinsic pathway

mechanism involves indirect activation of caspases by agents that induce DNA damage such as irradiation and cancer chemotherapeutic drugs and pharmacological agents such as staurosporin⁶⁷. Other stimuli that trigger the intrinsic pathway include loss of survival or trophic factors, toxins, hypoxia, oxidative stress and ischaemia-reperfusion⁶⁸.

These apoptotic triggers result in a drop in the mitochondrial transmembrane potential and outer mitochondrial membrane permeability. This increase in permeability results in a release of apoptotic factors such as cytochrome C, Smac/DIABLO (another caspase activator) and apoptosis inducing factor into the cytosol⁶⁹⁻⁷¹. Smac results in cytochrome C dependent caspase activation⁵⁷. DIABLO binds to Inhibitor of Apoptosis proteins (IAP) and thus indirectly activates the caspases⁷¹. Apoptosis inducing factor mediates apoptosis by both caspase dependent and caspase independent actions⁷⁰. Cytochrome C binds to Apoptosis protease- activating factor-1 which then interacts with caspase 9 and ATP to form the apoptosome^{47,72}. This subsequently triggers caspase 3 and 7 which eventually results in cell death. This has been schematically represented in **figure 1.06**.

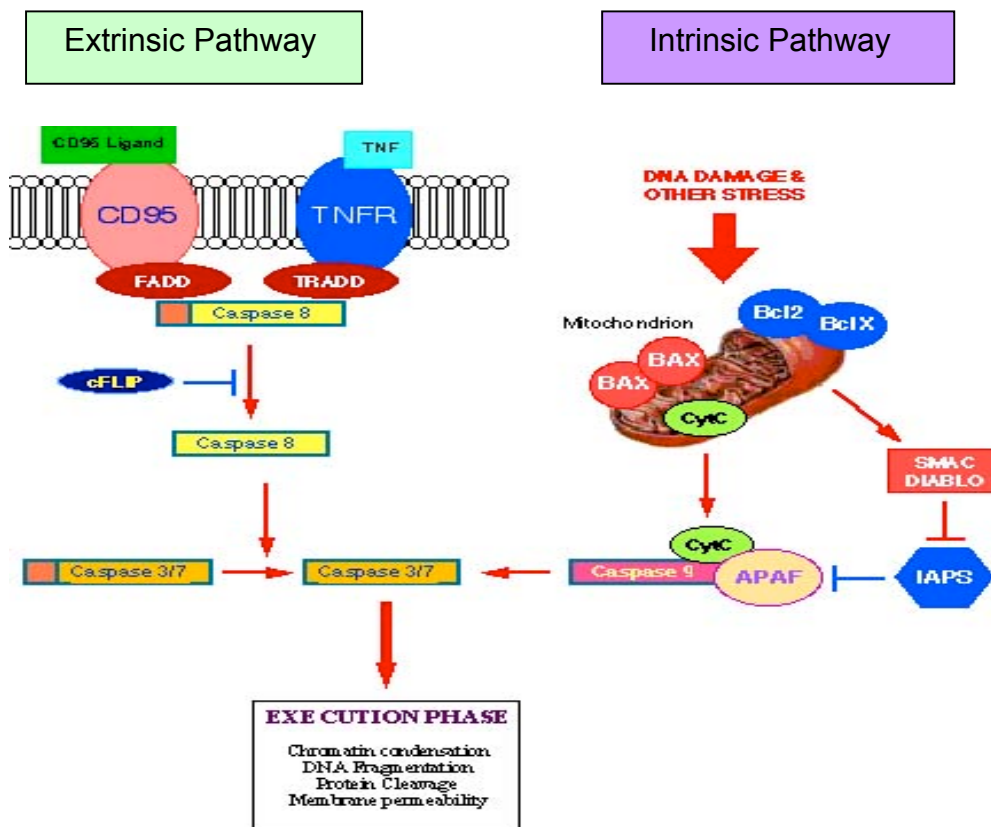


Figure 1.06: Mechanisms of Apoptosis: (a) The extrinsic pathway is mediated by binding of peptides like Fas/CD95 and Tumour Necrosis Factor- α (TNF- α) to the CD95 receptor and TNF receptor (TNFR) respectively. These then bind to caspase 8 which subsequently activates caspase 3/7. (b) The intrinsic pathway is triggered by hypoxia, ischaemia-reperfusion and irradiation and results in excessive outer mitochondrial membrane permeability and the release of Cytochrome C, SMAC/DIABLO and Apoptosis Inducing Factor into the cytosol. Cytochrome C binds to Apoptosis Protease Activating Factor (APAF) which then binds to caspase 9 and eventually activates caspase 3/7. SMAC/DIABLO bind to Inhibitors of Apoptosis Proteins (IAPs) and thus indirectly activates APAF/Cytochrome C/caspase 9. Adapted from www.apoptosisworld.com.³⁷⁷

The Outer Mitochondrial Membrane permeability is controlled by the Bcl2 group of proteins⁷³. The anti-apoptotic members Bcl2 and Bcl X_L inhibit the release of apoptotic factors and are usually membrane bound while the pro-apoptotic members such as Bax, Bak, Bad and Bid are present in the cytosol and translocate to the outer mitochondrial membrane, where they promote the release of these factors^{72,74}. Bid is cleaved by caspase 8 and binds to Bax or

Bak and ensures translocation of these on to the outer mitochondrial membrane, where they homo-oligomerize to form channels that are permeable to the apoptotic factors⁷⁵. Anti-apoptotic members such as Bcl-2 bind to Bak or Bax and prevent the conformational change and thus the formation of ion channels⁷⁶. Bad protein inhibits this action by binding to the Bcl2 protein and thus triggers apoptosis⁷².

The other mechanism of outer mitochondrial membrane permeability is the mitochondrial permeability transition pore (MPTP) and this is discussed in a subsequent section. The MPTP is not triggered by the Bax/Bak system and can be modulated by the Bcl-2 pro survival proteins and depending on the degree of ATP depletion is associated with either necrosis or apoptosis⁷³. These two mechanisms are illustrated in **figure 1.07**.

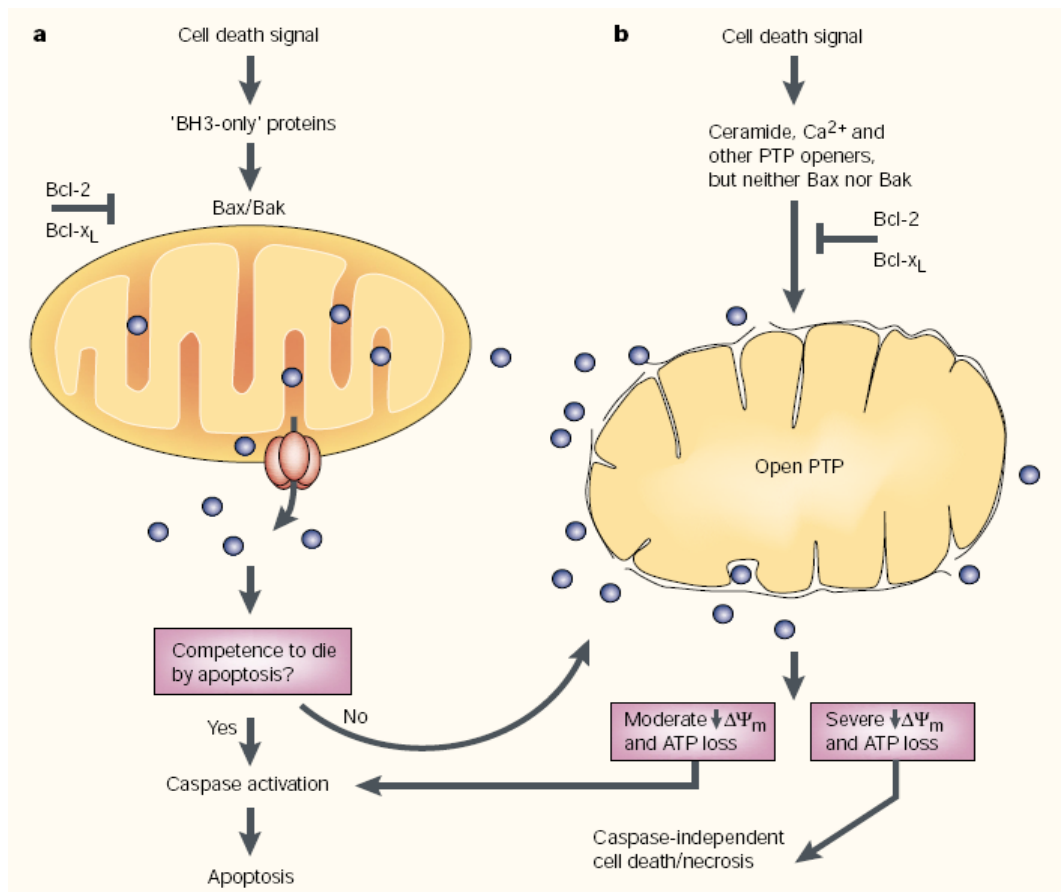


Fig 1.07: Outer Mitochondrial Membrane Permeability: (a) The induction of Bax/Bak results in increased outer membrane permeability to cytochrome c which results in caspase activation and cell death by apoptosis. The blue dots represent cytochrome c and apoptogenic factors and the channel in the outer mitochondrial membrane consists of oligomerized Bax proteins. (b) The formation of the mitochondrial permeability transition pore results in mitochondrial swelling. Depending on the degree of ATP depletion, the cells progress to a caspase independent necrotic cell death or a caspase dependent apoptotic cell death. Taken from Martinou et al.⁷³

CONTRIBUTION OF APOPTOSIS AND NECROSIS TO LETHAL REPERFUSION INJURY IN THE CARDIOMYOCYTES

While there is a lot of controversy regarding the percentage contribution of the two modes of cell death to reperfusion injury in the cardiomyocyte, it is becoming clear that both mechanisms are involved to varying extents⁶⁸. Apoptosis has been shown to contribute to about 13% of cardiomyocyte loss in the setting of rat heart ischaemia-reperfusion injury⁷⁷. Post mortem studies in patients with acute myocardial infarction treated with thrombolysis, has revealed

the presence of apoptosis in cardiomyocytes⁷⁸. This has been also seen to occur in remote viable myocardium following acute myocardial infarction⁷⁹. However these conclusions have been largely based on biochemical and immunohistochemical data. All morphological and ultrastructural data point towards necrosis as the predominant mode of death⁸⁰.

Mutant mouse models with mutations in various pro-apoptotic genes or ligands have shown a 50-60% reduction in myocardial infarct size. These mutations include mutation of the Fas ligand of the death domain, knockout of pro-apoptotic Bid and overexpression of antiapoptotic Bcl-2^{81,82}.

It has thus been hypothesized that cells undergo apoptosis and necrotic cell death early during ischaemia and then demonstrate morphological characteristics of necrosis once irreversibly injured⁶⁸. The other postulate is that cardiomyocytes undergo a hybrid form of cell death involving characteristics of both apoptosis and necrosis⁶⁸. The experiments in this thesis deal with infarction as a manifestation of necrosis.

1.2.3.2 VASCULAR ENDOTHELIUM AND LETHAL REPERFUSION INJURY

Lethal reperfusion-injury occurs due to changes in both the vascular endothelial cells and also the cardiac myocytes. However this thesis will primarily concentrate on the cardiac myocytes but I will briefly summarize the effect of reperfusion on the vascular endothelium.

Microvascular dysfunction is an early and rate determining factor in the pathogenesis of ischaemia-reperfusion injury⁸³. This is manifest as impaired endothelium dependent dilation in the arterioles, enhanced fluid filtration and leukocyte plugging in the capillaries and the trafficking of leukocytes and

plasma protein extravasation in the post capillary venules⁸³. The endothelial cells are very sensitive to ischaemia and endothelium dependent dilation is affected significantly during the first few minutes of reperfusion⁸⁴.

Endothelium associated dilation is dependent on nitric oxide synthase (NOS) which produces a potent vasodilator, nitric oxide (NO). Reperfusion results in the production of free radicals such as superoxide which can interact with NO to form peroxynitrite free radicals and thus reduce the bioavailability of nitric oxide and also result in endothelial damage⁸⁵. In a porcine model, it has been shown that addition of tetrahydrobiopterin (a cofactor of NOS) following ischaemia restores the availability of nitric oxide⁸⁶. L-arginine is an essential substrate for nitric oxide synthesis and in a feline model, addition of L-arginine has shown to preserve endothelial function⁸⁷. Reperfusion associated endothelial dysfunction is partly due to the upregulation of arginase (which results in the reduced bioavailability of arginine) by Tumour Necrosis Factor- α (TNF- α) as demonstrated in a mouse in-vivo model of ischaemia-reperfusion⁸⁸.

Endothelial and parenchymatous oedema along with the adhesion of neutrophils and platelets to the endothelial surface is responsible for the 'no reflow' phenomenon at the capillary level⁸⁹. Reperfusion activates the complement system and the C5b-9 complement complex directly inhibits endothelium-dependent vasodilation while C5a activates inflammatory mediators such as Interleukin-6 (IL6) and TNF α . C3b directly activates the binding of neutrophils to endothelium⁸⁹. These are the main events that are responsible for 'no reflow' at the capillary level.

The inflammatory response is responsible for injury that occurs at the post-capillary level. Chemotactic agents such as IL-6, TNF α , Platelet Activating

Factor (PAF) etc., promote the adhesion between neutrophils and the endothelium and subsequent migration of neutrophils to the parenchyma resulting in neutrophil degranulation and production of Reactive Oxygen Species (ROS) and thus endothelial and myocyte cell death⁸⁹.

TNF α also triggers endothelial apoptosis via activation of caspases and this in turn influences apoptosis in the adjacent parenchyma^{90,91}. Thus it is evident that endothelial cells have a role to play in the pathogenesis of reperfusion injury.

1.2.3.3. EVENTS IN THE CARDIOMYOCYTE

Lethal reperfusion-injury predominantly occurs within the first few minutes of the onset of reperfusion and is thought to be mediated by mitochondrial related mechanisms⁹². In addition, a delayed reperfusion injury is also thought to occur in vivo, which is mediated by polymorphonuclear cells that infiltrate the infarcted territory after 24 hours and result in apoptotic cell death⁷.

It has also been shown that various measures to attenuate infarct extension as a result of reperfusion injury are most effective in early reperfusion. The following changes occur in the cardiomyocyte during the first few minutes of reperfusion and the mechanisms targeting these phenomena can result in cardioprotection:

(a)**Oxygen and calcium Paradox**: During ischaemia the Na⁺/K⁺ ATPase on the sarcolemmal membrane and the Ca²⁺ ATPase in the sarcoplasmic reticulum become inactive as the energy stores are depleted. This results in accumulation of sodium and calcium in the cytoplasm. However during ischaemia the Na⁺/Ca²⁺ exchanger which is energy independent is active in its attempt to rid

the cell of excess sodium and results in worsening of the intracellular calcium concentration.

However during the process of reperfusion, the return of oxygen results in re-energisation of energy dependent Na^+/K^+ ATPase and Ca^{2+} ATPase pumps. This paradoxically worsens the intracellular calcium levels (discussed below) that results in hypercontracture and cytoskeletal disruption and cell death. This is termed the 'oxygen paradox'. Those cells that have irreversible damage to these channels during ischaemia are unable to correct the cation imbalance and thus progress to cell death without being affected by reperfusion injury.

The cardiomyocytes that have functional sarcolemmal Na^+/K^+ ATPase and SR Ca^{2+} ATPase respond to re-energisation. Restoration of Na^+/K^+ ATPase activity results in a trans-sarcolemmal sodium gradient that is sufficient to activate the $\text{Na}^+/\text{Ca}^{2+}$ exchanger in the 'forward mode' i.e., extruding calcium for sodium. The Ca^{2+} ATPase activation results in accumulation of calcium within the sarcoplasmic reticulum leading to oscillatory calcium movement between the SR and the cytoplasm until the excessive calcium has been extruded from the cytoplasm. However the high levels of calcium that have accumulated within the myocyte during ischaemia and subsequently due to the oscillatory activity achieved during reperfusion, results in myofibrillar hypercontracture with the restoration of energy. This results in brittle cytoskeletal architecture which in turn compromises the cellular integrity^{7,93}. These phenomena are illustrated in **Figure1.08**.

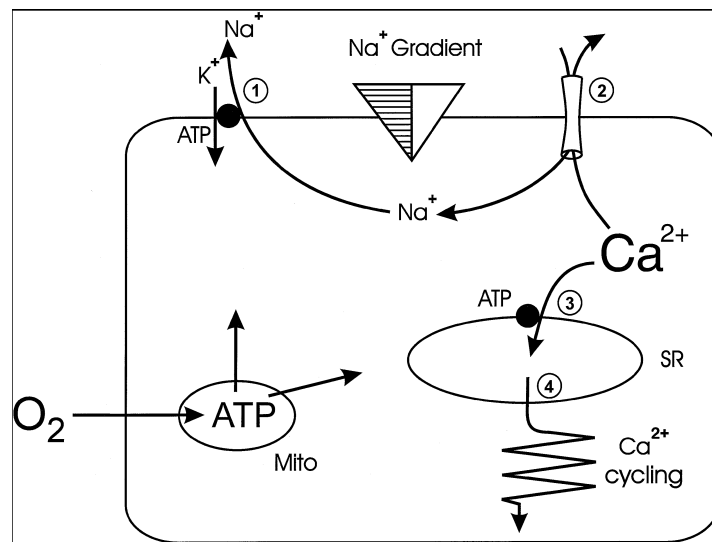


Figure 1.08: Reoxygenation results in activation of the Na/K ATPase (1) which in turn results in activation of the Na⁺/Ca²⁺ exchanger (2). This results in activation of the SR Ca²⁺ ATPase (3) which eventually contributes to oscillatory calcium levels (4) and thus myofibrillar contracture. Taken from Piper et al⁷.

(b) **pH Paradox:** Ischaemia triggers anaerobic glycolysis which in turn results in a depletion of ATP. This results in profound intracellular acidosis. However during reperfusion there is rapid normalization of the extracellular pH and this creates a H⁺ gradient. Furthermore reenergisation activates the H⁺ extruding mechanisms at the level of the sarcolemmal membrane. These include the Na⁺/H⁺ exchanger and the Na⁺/HCO₃⁻ symporter. The intracellular acidosis during ischaemia actually inhibits the responsiveness of the myofibrillar machinery to calcium.

The correction of acidosis during reperfusion essentially reverses this inhibitory action and a responsive myofibrillar machinery in the presence of a calcium load, results in hypercontracture. Of further importance is the fact that the accumulation of intracellular Na⁺, due to activation of the H⁺ extruding mechanisms, results in a reverse mode switch of the Na⁺/Ca²⁺ exchanger and thus increases the levels of intracellular calcium. This results in excessive

calcium load and hypercontracture^{7,93}. These have been elucidated in **Figure1.09**.

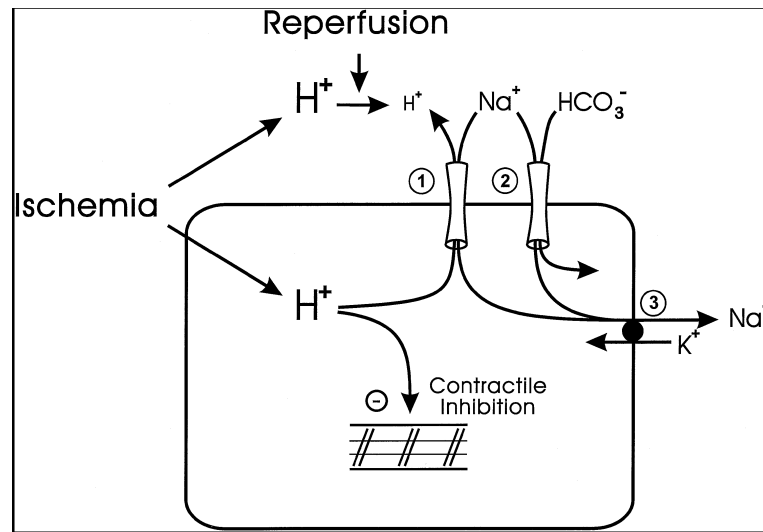


Figure 1.09: During ischaemia intracellular acidosis results in inhibition of myofibrillar machinery. Correction of extracellular acidosis during reperfusion, triggers the extrusion of H^+ and thus sets in motion myofibrillar contracture in the presence of excess calcium. Taken from Piper et al⁷.

(c) **Increase in Osmolarity:** The other crucial change that results in cell death is the increase in tissue osmolarity. During ischaemia there is an increase in the intracellular osmolarity due to accumulation of intracellular Na^+ . However the presence of hyperosmolar metabolites in the extracellular space inhibits any osmotic fluid overload within the cell and thus the cell does not swell up. The intracellular accumulation of Na^+ occurs because of the inhibition of the Na^+/K^+ ATPase and also the activation of the Na^+/H^+ exchanger in an effort to maintain the intracellular pH during ischaemia.

Reperfusion ensures a wash out of the osmotic metabolites in the extracellular space and results in an osmotic gradient that results in cellular and sarcolemmal swelling. However this in itself is not sufficient to cause cell rupture. Reoxygenation and the increased calcium load associated with reperfusion results in an increase in the structural fragility of the cell and thus

cell rupture occurs because of the associated oedema. It has also been demonstrated that there is the activation of proteolytic agents called calpains during reperfusion. These enzymes result in the proteolysis of ankyrin filaments that are responsible for the anchorage of the Na^+/K^+ ATPase to the sarcolemmal membrane. This worsens the intracellular Na^+ load and thus further osmotic swelling occurs^{7,93}.

(d) **Reactive Oxygen Species:** Reperfusion also results in the formation of Reactive Oxygen Species (ROS). It was first demonstrated in perfused rat hearts that the use of free radical scavenger DMSO resulted in a reduction in CK release⁹⁴. Experiments performed in rabbit hearts also showed that free radical production peaks at 15-20 seconds of reperfusion and this was associated with alterations in the recovery of high energy phosphate metabolism⁹⁵. A 'burst' of H_2O_2 and hydroxyl radicals was seen to occur during the first 5 minutes of reperfusion and antioxidants were shown to improve cell viability in an isolated cardiomyocyte model⁹⁶. Apart from antioxidants, ischaemic preconditioning and hypothermia have also been shown to inhibit this burst⁹⁷. This ROS burst also triggers the induction of the mitochondrial permeability transition pore which in turn generates more ROS⁹⁸. These detrimental ROS largely tend to be singlet oxygen species, superoxide or peroxynitrite. Peroxynitrite ROS results in lipid peroxidation and loss of cellular integrity^{7,93}.

Some ROS can also be beneficial in that they play a regulatory role in energy metabolism, apoptosis signalling, necrosis, ion channel regulation and vascular tone⁹⁹. Experiments in rabbit hearts have shown that antioxidants abrogate the infarct reduction of ischaemic preconditioning thus suggesting a beneficial role for ROS in preconditioning¹⁰⁰. Similar results have been obtained in

cardiomyocytes¹⁰¹. The mitochondrial K⁺ ATP channels are central to this beneficial role in preconditioning¹⁰². However excessive amount of ROS is detrimental to cell survival.

(e) **Inflammation**: Various inflammatory interactions that occur later as a result of neutrophil activation and accumulation contributes to reperfusion injury^{103,104}. Neutrophil adhesion to the coronary vascular endothelium occurs within minutes of reperfusion and is associated with progressive endothelial dysfunction¹⁰⁵. The neutrophils are present in the intravascular space for the first 6 hours of reperfusion and then migrate transendothelially into the parenchyma over the ensuing 24 hours and this is responsible for the delayed phase of reperfusion injury¹⁰⁶.

The triggers of neutrophil activation include cytokines such as tumour necrosis factor- α (TNF α), Interleukin6 (IL6), platelet activating factor and complement C5a which are released by the ischaemic perfused myocardium and regulated by the expression of NF- κ B and ROS^{107,108}. ROS such as the superoxide anions, hydroxyl radicals and hydrogen peroxide activate neutrophils by upregulating the adhesion molecules such as ICAM and p-selectin and also the activation of cytokines^{109,110}. Lipid mediators like leukotriene B4, thromboxane A2 and Platelet Activating Factor are also involved in chemoattraction of neutrophils¹⁰⁴.

The binding of neutrophils to the endothelium is dependent on p-selectin. Subsequent rolling is dependent on β 2 integrins such as CD11/CD18. The CD 18 molecule binds to ICAM-1 adhesion molecule on the endothelium and subsequent neutrophil migration to the interstitium occurs as a result of binding

to PECAM. The neutrophils then bind to the myocyte via ICAM and releases proteases and cytotoxic oxidants¹⁰⁴.

Neutrophils play a role in cardiomyocyte death by one or many of the following mechanisms: (a) The release of ROS while interacting with the endothelium prior to transendothelial migration¹¹¹. (b) release of proteases and collagenase. (c) microvascular embolization and endothelial dysfunction¹¹². (d) Triggering apoptosis and infarct size extension in the late phase of reperfusion¹¹³.

Various measures to reduce neutrophil associated reperfusion injury, have been used. Some of these include (a) Leukocyte depletion using neutrophil antiserum¹¹⁴. (b) Selective antibody therapy to adhesion molecules like anti-selectin antibodies¹¹⁵ and monoclonal antibodies to the integrins CD11/CD18¹¹⁶. (c) Adenosine which is known to have anti neutrophil activity by virtue of reducing superoxide formation¹¹⁷. This has been shown to be due to its activity on the A2A receptor¹¹⁸. (d) Local anaesthetics like lidocaine have an effect on infarct size by interacting with various mechanisms of neutrophil activation¹¹⁹.

(f) **The Mitochondrial Permeability Transition Pore (MPTP)**: The MPTP is a non specific pore that is present on the inner mitochondrial membrane which opens in response to the combination of calcium load, oxidative stress, pH change and ATP depletion associated with ischaemia-reperfusion injury. This uncouples oxidative phosphorylation and results in depletion of cellular ATP levels which in turn results in necrotic cell death¹²⁰. The role of the pore in lethal reperfusion injury is discussed in the next section.

1.2.3.4. THE MITOCHONDRIAL PERMEABILITY TRANSITION PORE

The MPTP is a non specific pore that remains tightly closed under normal physiological conditions and is present on the inner mitochondrial membrane¹²⁰. When open it is permeable to any molecule which is less than 1.5 kDa and usually results in hydrolysis of ATP and rapid depletion in energy stores¹²¹.

It is not entirely clear as to what constitutes the pore though its electrophysiological and pharmacological characteristics have been determined by various experiments. It has been demonstrated in isolated rat heart mitochondria, that administration of cyclosporin A inhibits pore opening and that this is due to the inhibition of the activity of a matrix peptidyl-prolyl cis-trans isomerase known as cyclophilin D¹²². However it would appear that cyclophilin D aids in conformational change of an as yet unidentified protein under the influence of calcium¹²⁰.

The adenine nucleotide translocase (ANT) has also been shown to undergo conformational change and activate the MPTP in isolated rat heart and liver mitochondria¹²². ANT is activated by oxidative stress and inhibits the binding of adenine nucleotides to the cyclophilin D moiety in isolated rat heart mitochondria¹²³. However experiments in mouse liver mitochondria have shown that genetic ablation of ANT has no effect on the ability for cyclosporin A to inhibit pore opening, although a higher concentration of calcium was required for the activation of the pore¹²⁴.

More recently a mitochondrial phosphate carrier has been implicated to be a component of the pore and its binding to cyclophilin D was shown to be inhibited by ubiquinone analogues (inhibitors of the pore), in rat heart mitochondria¹²⁵.

MPTP opening results in neutralization of the mitochondrial transmembrane potential ($\Delta\Psi_m$) and inhibition of ATP synthesis⁹². This is associated with influx of water and mitochondrial swelling which results in the failure of the outer mitochondrial membrane and cell death.

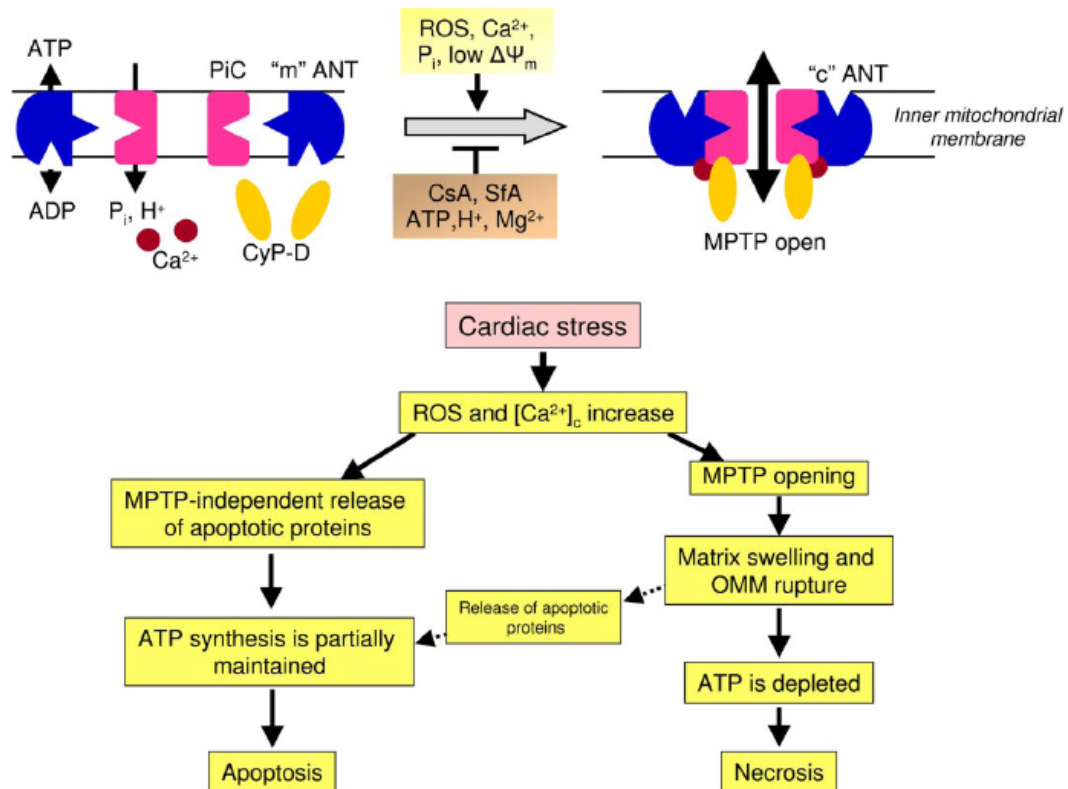


Fig 1.10: Components and formation of the MPTP and subsequent events. ANT: Adenine nucleotide translocase, PiC: Phosphate carrier, Cyp D: Cyclophylin D, CsA: Cyclosporin A and SfA: Sangliferin A. Taken from Javadov et al¹²⁶.

The pore can be inhibited by direct inhibitors such as cyclosporine, sangliferin A and ANT inhibitors such as bongkreikic acid¹²⁷⁻¹²⁹ and indirectly by ROS scavengers such as propofol, low pH inducers such as the Na⁺/H⁺ exchange inhibitor-cariporide, ubiquinone analogues and preconditioning/postconditioning¹³⁰⁻¹³².

The pore remains shut during ischaemia but opens 2-3 minutes into reperfusion when the pH has normalised¹³³. Further evidence that the pore is associated with reperfusion injury was obtained when sangliferin A and cyclosporine A

were shown to protect the heart from reperfusion injury^{127,134}. MPTP opening is activated by ROS, increased calcium which occurs due to inhibition of oxidative phosphorylation, the rapid correction of pH during reperfusion and ATP depletion^{30,92,130,135}. This is represented in **Figure 1.10**.

The various changes that occur with reperfusion injury can be altered by various direct and indirect mechanisms. The signal transduction mechanisms that can potentially attenuate reperfusion injury will be the subject of the next section.

1.3 THE REPERFUSION INJURY SALVAGE KINASE (RISK) PATHWAY

The Reperfusion Injury Salvage Kinases are a specific group of proteins that are activated in the reperfusion phase and confer cardioprotection by preventing lethal reperfusion injury³⁰. These are the so called pro-survival anti-apoptotic kinases and include PI3K/Akt and ERK1/2¹³⁶. There are various targets for these pro-survival kinases which have been elucidated by studying the effect of pharmacological manipulation of these kinases and this will be discussed in a separate section. The RISK pathway now also includes cardioprotective reperfusion salvage kinases such as Protein Kinase C- ϵ (PKC- ϵ) isoform, Protein Kinase G (PKG), p70s6K and GSK 3 β ¹³⁷. The RISK pathway and its effectors however represent a core component of a common cardioprotective pathway that terminates on the MPTP and unites preconditioning, postconditioning and pharmacological manipulation of reperfusion injury¹³⁸. RISK pathway activation should occur in the first few minutes of reperfusion in order to be of any benefit.

Necrosis and apoptosis contribute to myocardial reperfusion injury to different extents as already discussed⁴⁵. However the contribution of necrosis to

reperfusion injury is significantly higher⁴⁵. The prosurvival kinases that form part of the RISK pathway activate various downstream effectors that interact with both apoptotic cell death and necrotic cell death.

1.3.1 COMPONENTS OF RISK PATHWAY AND ITS EFFECTORS

1.3.1.1 PHOSPHATIDYLINOSITIDE-3-KINASE (PI-3K)

The first link in the RISK pathway is the phosphatidylinositide-3-kinase (PI-3K). PI-3Ks are a family of evolutionary conserved lipid kinases that mediate many cellular responses in both physiological and pathological states¹³⁹. PI3Ks are divided into three classes based on substrate specificity, mode of action and molecular structure. These are PI3K-1A, PI3K-1B, PI3K-2 and PI3K-3¹³⁹. Class 1 PI3Ks and class 3 PI3Ks are heterodimeric and consist of a catalytic and accessory subunit while class 2 PI3Ks are monomeric¹⁴⁰. Class 1A kinases bind to the monomeric G protein ras and participate in phospholipid binding and protein-protein interaction while class 1B bind to G protein coupled receptors (GPCR)¹⁴¹. This eventually results in activation of tyrosine kinases and downstream effectors that mediate the various effects of PI3K.

The molecular targets of PI3K are PDK-1 (Phosphoinositide Dependent Kinase-1), Akt/PKB, GSK-3 β , mTOR, p70S6 kinase and Rho GTPases¹³⁹. PDK-1 is an ubiquitously present 67 KDa kinase. PDK-1 is central in the PI3K signalling pathway and deletion of cardiac specific PDK-1 results in reduced cardiac size, cardiomyopathy and increased sensitivity to hypoxia¹⁴². PDK-1 phosphorylates other kinases like the AKT/PKB, Protein Kinase C (PKC) and p70S6 kinase¹⁴³.

PI3K mediated AKT/PKB and GSK 3 β actions will be discussed in the next section. mTOR and p70S6 kinase are predominantly involved in cardiomyocyte growth.

1.3.1.2 Akt/PKB

Akt/PKB is a serine/threonine protein kinase and comprises of 3 closely related members which are Akt1, Akt2 and Akt3¹⁴⁴. Akt appears to play an important role in majority of the cardioprotective actions¹⁴⁵. PI3K activation results in the formation of phosphoinositol 3 phosphate (PIP3) which in turn brings about a translocation of Akt to the cell membrane. Akt is then activated by phosphorylation at threonine 308 site by PDK-1 and is regulated at the serine473 site by TORC2^{146,147}. The targets of Akt include FOXO transcription factors, caspases, GSK-3 β and the pro-apoptotic Bcl-2 family protein BAD^{65,136}. PI3K activation enhances cell survival and antagonizes apoptosis by Akt/PKB activation^{148,149}.

The following mechanisms of Akt mediated cardioprotection are relevant in the setting of ischaemia-reperfusion injury:

(a) Akt phosphorylates the pro apoptotic proteins BAX and BAD. This results in its inactivation and intracytoplasmic sequestration and prevents it from translocating on to the mitochondrial membrane, the action of which has been shown to inhibit the opening of MPTP^{136,150}. Akt agonists have been shown to inhibit doxorubicin induced reduction in the anti- apoptotic protein Bcl X_L in cardiomyocytes and this results in caspase 3 inactivation¹⁵¹. Preconditioning which triggers Akt activation has been shown to prevent ischaemia-reperfusion associated reduction in Bcl2 expression^{152,153}. Akt inhibits caspase9 and caspase3 both of which are potent mediators of apoptosis¹⁵³.

(b) Akt phosphorylation of GSK 3 β (Glycogen Synthase Kinase-3 β) at Serine 9 results in the inactivation of this protein¹⁵⁴. The exact downstream effector of GSK 3 β is not entirely clear, however it has been shown that inactivation of GSK 3 β results in delay of the opening of the MPTP¹⁵⁵. Nishihara et al did demonstrate that phosphorylation of GSK 3 β in response to preconditioning and erythropoietin treatment resulted in a decrease in the association of ANT and cyclophilin D and thus resulted in inhibition of pore opening¹⁵⁶. It has also been suggested that it indirectly inhibits MPTP opening by reduction in the amount of reactive oxygen species (ROS) release and also its interaction with the PKC (Protein Kinase C) domain of the mitochondrial ATP dependent potassium channel (Mito-K-ATP)^{136,155,157}. GSK 3 β inhibition is also known to occur via activation of p70s6k¹⁵⁵. PI3K also enhances glucose uptake by an Akt dependent translocation of the insulin dependent GLUT4 transporters to the plasma membrane and increases glycogen synthesis by Akt mediated GSK inhibition^{158,159}.

However it has recently been shown that mice hearts with GSK double knockin inhibition did not attenuate the ability to pre or post-condition these hearts¹⁶⁰. It thus remains unclear as to whether phosphorylation of GSK 3 β is involved in Akt dependent inhibition of pore opening.

(c) Endothelial Nitric Oxide Synthase (eNOS) is another downstream effector of Akt. It may have the potential to inhibit MPTP opening either through its activity on PKG-PKC ϵ -mito KATP channel¹⁶¹ or through the production of Nitric oxide which eventually activates PKG, an inhibitor of MPTP opening¹⁶².

(d) Hexokinase-2 is expressed in cardiomyocytes and has been shown to be phosphorylated by Akt in neonatal rat ventricular myocytes¹⁶³. The dissociation

of hexokinase-2 from the outer mitochondrial membrane has been associated with increased susceptibility of mitochondria to ROS induced activation of MPTP, in adult cardiac myocytes¹⁶⁴. It is proposed that the preferential availability of ATP to hexokinase results in a constant supply of ADP to the mitochondria and this is responsible for its resistance to ROS induced depletion in ATP¹⁵⁴.

(e) Akt is also known to activate cGMP and PKG which phosphorylates phospholamban and thus has an effect on Ca²⁺ uptake of the sarcoplasmic reticulum. This is mediated via eNOS. This in turn has been shown to inhibit the MPTP opening^{136,165,166}.

1.3.1.3 EXTRACELLULAR SIGNAL REGULATED KINASE 1&2 (ERK1/2)

ERK1 & 2 belong to the family of proteins called Mitogen Activated Protein Kinases (MAPKs)¹⁶⁷. The MAPKs are a family of serine/threonine protein kinases whose function and regulation has been evolutionarily conserved¹⁶⁸. MAPKs regulate a wide range of functions including cell growth and differentiation, gene expression, mitosis, cell motility, metabolism, cell survival and apoptosis and embryogenesis¹⁶⁹. ERK1 & 2 consists of a 42 and a 44 KDa kinase (p42 & p44)¹⁷⁰. ERK1/2 are activated by the MAP kinase/ERK kinase (MEK1/2) which in turn are activated by small G proteins ras-raf¹⁶⁷. Protein Kinase C α (PKC α) has also been shown to phosphorylate raf and thus activate ERK1/2¹⁷¹.

Various cardioprotective agents and mechanisms have been shown to activate ERK1/2. Postconditioning has been shown to activate ERK1/2 in isolated buffer perfused rabbit heart but not PI3K/Akt signalling¹⁷². Primary cardiac myocytes when exposed to urocortin have been shown to protect the cells from

reperfusion injury and this is associated with rapid phosphorylation of ERK1/2¹⁷⁰. Urocortin is a peptide related to the hypothalamic corticotrophin-releasing hormone and binds with high affinity to the corticotrophin –releasing hormone receptor 2 β which is present in the heart¹⁷⁰. Administration of erythropoietin, both in vitro and in vivo rat heart models, has been shown to reduce infarct size and this paralleled the activation of ERK1/2¹⁷³.

ERK1/2 has been shown to inhibit apoptosis by inactivating the proapoptotic BAD and this is dependent on phosphorylation of p90rS6 kinase or p70S6 kinase^{174,175}. ERK1/2 also promote cell survival by phosphorylating and stabilizing the expression of the anti apoptotic protein Mcl1¹⁷⁶. It also inhibits caspase 9 activity and thus regulates apoptosis¹⁷⁷.

The various mechanisms associated with RISK pathway activation have been illustrated in **figure 1.11**.

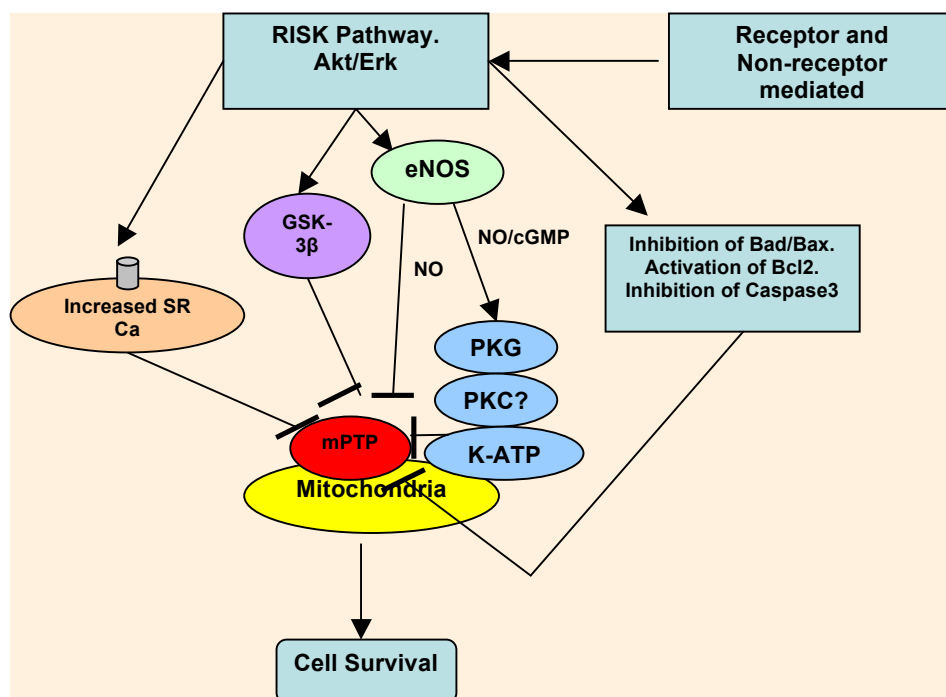


Fig 1.11: Various mechanisms of RISK pathway mediated cell survival. Adapted from Hausenloy et al¹³⁶.

Various pharmacological agents have been shown to activate the RISK pathway and this has opened up a plethora of potential cardioprotective agents that can be potentially used as adjuncts in reperfusion.

1.3.2 MYOCARDIAL PROTECTION BY ADJUNCTS USED AT REPERFUSION

A variety of adjuncts have been shown to protect the heart from reperfusion injury by recruiting the RISK pathway. The list of agents is constantly evolving but only a few of these have translated into viable agents in human trials. Some of these agents are discussed below.

(a) **Adenosine**: Adenosinergic mechanisms have long been established as central to ischaemic preconditioning. However it is increasingly becoming clear that it is also central to postconditioning and pharmacological attenuation of reperfusion injury. Adenosine has 4 different receptor targets which are adenosine receptor isoforms: A₁, A_{2A}, A_{2B} & A₃. It has been shown in various studies that adenosine agonists when administered during reperfusion acts via the A₁ and A_{2B} receptors and activates the PI3 Kinase and MEK1/2 pathway to eventually activate the mito K⁺-ATP channels^{178,179}. It has also been shown that activation of the A₃ receptor results in infarct sparing through GSK3 β and direct inhibition of MPTP¹⁶⁶. Disappointingly the clinical trials utilizing adenosine in Acute Myocardial Infarction have not shown a similar infarct sparing effect^{180,181}. This has been attributed to the fact that infarct size measurement mechanisms are not robust enough in clinical trials¹⁶⁶. The role of adenosine in cardioprotection has been discussed in more detail in a separate section.

(b) **Metformin**: The results of UKPDS34¹⁸² trial suggested that Metformin offered benefits to diabetic patients who are overweight with ischaemic heart disease. In the UKPDS study 1704 patients were recruited and randomly assigned to conventional diet control, intensive therapy with sulphonylurea and insulin or Metformin alone. It was noted that the incidence of myocardial infarction was 36 % less in the Metformin group when compared to the conventional treatment group. However the glycaemic control was similar in both the treatment groups and this may suggest that Metformin has additional cardioprotective effects apart from its antihyperglycaemic actions.

Metformin has been shown to improve cardiac function in streptozotocin diabetic rats in an isolated rat heart model¹⁸³. It has been shown to reduce infarct size in isolated perfused rat hearts subjected to global ischaemia¹⁸⁴. Further insight into the mechanism of action was obtained by experiments in the isolated rat heart model and in cardiac myocytes, wherein metformin administered at reperfusion was able to reduce infarct size in both diabetic and non-diabetic hearts and this was shown to be dependent on PI3k/AKT inhibition of MPTP opening¹⁸⁵.

In bovine aortic endothelial cells metformin has been shown to improve endothelial function by PI3K mediated activation of AMP activated protein kinase (AMPK)¹⁸⁶. In the same study this was shown to be dependent on the phosphorylation of eNOS and increase in the bio-availability of nitric oxide. AMPK is a phylogenetically conserved heterotrimeric protein that is activated by pathological stresses such as glucose deprivation, hypoxia, oxidant stress and osmotic shock¹⁸⁷. In a mouse in vivo model Metformin has been shown to reduce infarct size when administered prior to ischaemia and at the onset of reperfusion and this effect is dependent on activation of AMPK and eNOS¹⁸⁸.

Recent data from a rat model of myocardial infarction (ex vivo and in vivo) has shown that acute cardiovascular protection of Metformin administered at reperfusion, was dependent on adenosine receptor stimulation probably via the increase in intracellular adenosine concentration¹⁸⁹. This may be a potential explanation for AMPK associated activation of eNOS as AMPK increases the availability of AMP, the concentration of which is a critical determinant of the endogenous adenosine pool¹⁸⁹.

(c) **Adipocytokines**: Adipose tissue is now recognised to be an organ with endocrine activity. It has been shown to secrete numerous hormones that have been collectively termed adipocytokines. Leptin is an adipocytokine that was originally discovered to be associated with obesity and the metabolic syndrome. It has since been demonstrated in an ex vivo animal model to attenuate infarct size when administered during reperfusion. This is due to activation of the RISK pathway and inhibition of the opening of the MPTP¹⁹⁰. Apelin another adipocytokine has also been shown to be directly cardioprotective during reperfusion by activating the RISK pathway¹⁹¹. Similar acute cardioprotection has been demonstrated with adiponectin and visfatin^{192,193}, suggesting a general protective effect of these agents that activate the RISK pathway.

(d) **Cyclosporin A**: Cyclosporin A is an immunosuppressive drug which is thought to inhibit the opening of the MPTP by preventing the binding of cyclophilin D to ANT and this has been shown to occur at very low concentrations¹⁹⁴. Cyclosporin A was first shown to be cardioprotective in an isolated rat heart model when administered prior to ischaemia¹²⁸. This protection was manifest as an improvement in left ventricular developed pressure (LVDP) and also better ATP/ADP ratios¹²⁸. Subsequently Hausenloy et al demonstrated in an isolated rat heart model that cyclosporin administered

at reperfusion reduced infarct size and that this was related to the inhibition of the MPTP¹⁹⁵.

In a small proof of concept clinical trial cyclosporin was administered to 58 patients prior to PCI for an acute ST elevation myocardial infarction. Troponin I and CK levels were used as surrogate markers of myocardial injury and in one subgroup cardiac MRI was used to assess infarct size. The release of CK and the absolute mass of infarcted tissue (MRI assessment at day 5 post myocardial infarct) was significantly lower in the Cyclosporin group¹⁹⁶.

(e) **Insulin**: Insulin is one of the first pharmacological adjuncts that was shown to be cardioprotective when administered during reperfusion. It has been shown to be protective in animal studies and in clinical studies^{197,198}. This has been demonstrated to involve the RISK pathway and the endothelial nitric oxide synthase and is believed to be predominantly an antiapoptotic effect¹⁹⁹. However administration of High dose Glucose Insulin Potassium (GIK) in patients with ST Elevation Myocardial Infarction as part of the CREATE-ECLA trial did not shown any cardiovascular benefit. This study was carried out with 7510 patients²⁰⁰. As a treatment agent its use is complicated by the risk of hypoglycaemic episodes.

(f) **Rho Kinase Inhibitors (Fasudil)**: Rho kinase is a G protein coupled kinase which is activated by the Rho A protein. Rho kinases are stimulated by hypoxia and vasopressors like angiotensin and are negative regulators of PI3 kinase/Akt/eNOS pathway^{201,202}. Rho kinase inhibition with administration of Fasudil at reperfusion has been shown to be cardioprotective in rat hearts and this protection was ameliorated by the administration of Akt and eNOS inhibitors²⁰³. The downstream targets of the Rho kinases still remains to be

elucidated, however it is speculated that this could be caspase3 mediated activity or Akt dependent maintenance of the balance between pro apoptotic and antiapoptotic mechanisms via Bcl2 regulation^{204,205}. Rho kinases have been described in more detail in a separate section.

(g) **Erythropoietin:** Erythropoietin (EPO) is a hormone that is produced in the juxtatubular cells of the kidneys and has a regulatory function with respect to erythropoiesis and is currently of therapeutic value in treating anaemia of chronic disease¹⁷³. However in a rat in vivo model EPO has been shown to attenuate infarct size in the heart when administered prior to reperfusion and this was associated with reduced DNA fragmentation in rat cardiac myoblasts exposed to hypoxia and reoxygenation²⁰⁶. Administration of darbepoietin α (a long-acting analog of EPO) prior to ischaemia-reperfusion has been shown to protect via MAPK/mitochondrial K-ATP channel dependent mechanism in an in vivo rat heart model²⁰⁷.

EPO has also been shown to protect cardiomyocytes from ischaemic injury and this is mediated by akt²⁰⁸. In a cardiomyocyte model EPO has been shown to be protective and this is related to upregulation of eNOS and NO²⁰⁹. EPO administered at reperfusion has also resulted in infarct size reduction in both isolated rat heart and an in vivo rat heart model and this has been shown to be dependent on PI3k and ERK¹⁷³. Similarly EPO administered at reoxygenation has been shown to be protective in a human atrial trabecular model of hypoxia-reoxygenation and this effect is dependent on PI3K/AKT and ERK1/2 activation and a reduction in caspase3 activation²¹⁰.

(h) **3-Hydroxy-3- Methyl Glutaryl CoA reductase (HMG CoA) Inhibitors**

(Statins): Statins are a group of drugs that are involved in the inhibition of the cholesterol biosynthetic pathway¹⁴. They are used in the treatment of hyperlipidemia and also in primary and secondary prevention of Ischaemic Heart Disease²¹¹. Statins have also been shown to have a number of other actions not related to its cholesterol lowering activity and these have been collectively termed pleiotropic actions¹⁴. These pleiotropic actions are the subject of this thesis and a brief review of this will form the basis of the next chapter.

1.4 STATINS, CHOLESTEROL AND CARDIOPROTECTION

Elevated levels of plasma cholesterol have long been established as an independent risk factor for atherogenicity and thus ischaemic heart disease. This has been exemplified by the Framingham study and the Multiple Risk Factor Intervention Trial (MRFIT) study²¹². While dietary cholesterol can affect the serum cholesterol levels, hepatic biosynthesis is the major source of serum cholesterol.

The rate limiting enzyme in the cholesterol biosynthetic pathway is the 3-Hydroxy 3-Methyl Glutaryl CoA reductase (HMG CoA reductase) and this enzyme catalyses the conversion of HMG CoA to mevalonic acid²¹³. Mevalonic acid is a common precursor for both cholesterol biosynthesis and the synthesis of isoprenoids such as farnesyl pyrophosphate and geranylgeranyl pyrophosphate. The isoprenoid geranylgeranyl pyrophosphate is required for activation of Rho group of proteins. This has been schematically represented in **figure 1.12**.

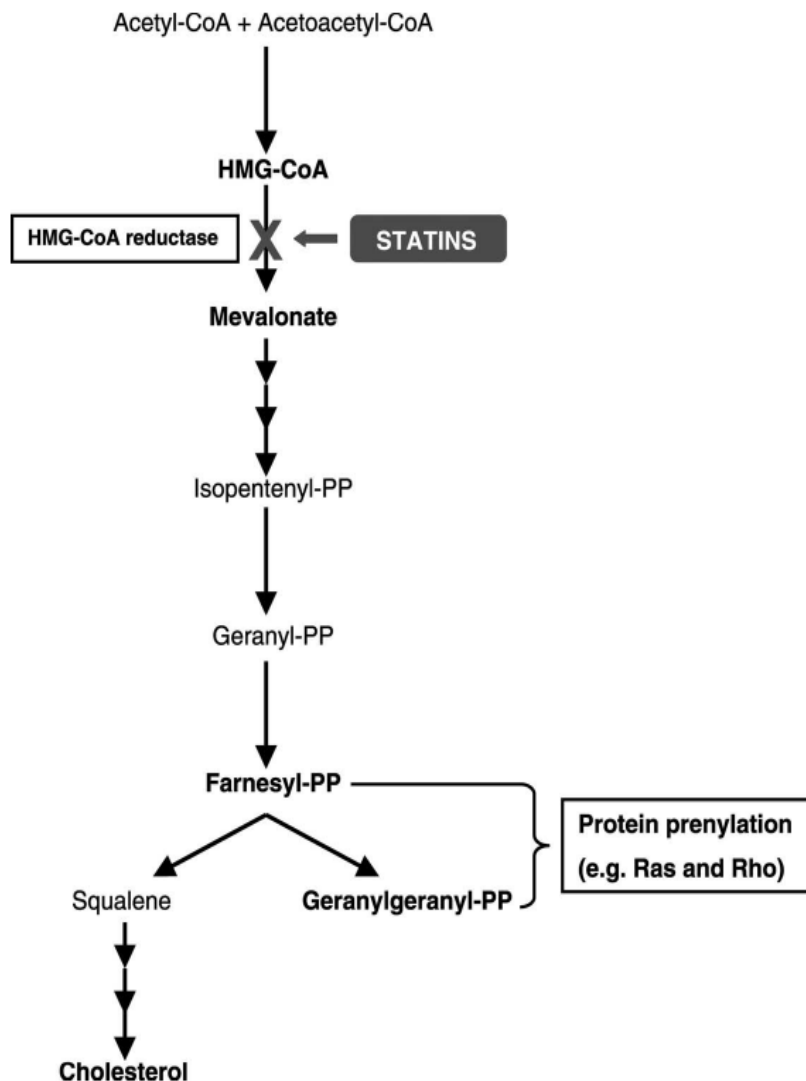


Figure 1.12: Cholesterol and Isoprenoid synthetic pathway (Cardiomyocytes). Taken from Bonetti et al²¹⁴.

The first statin to be discovered was isolated from the fungus *Penicillium citrinum* and was called Mevastatin²¹⁵. However this did not enter clinical use due to its hepatocellular toxicity. The first statin to gain approval for clinical use was lovastatin which was isolated from *Aspergillus terreus*²¹⁶. Since then numerous statins both chemically modified and naturally occurring have been commercially available. These include Pravastatin, Simvastatin, Atorvastatin, Cerivastatin, Fluvastatin, Pitavastatin and Rosuvastatin¹⁴. Atorvastatin and Rosuvastatin are completely synthetic molecules²¹⁷. The structure of some of the statins has been represented in **figure 1.13**.

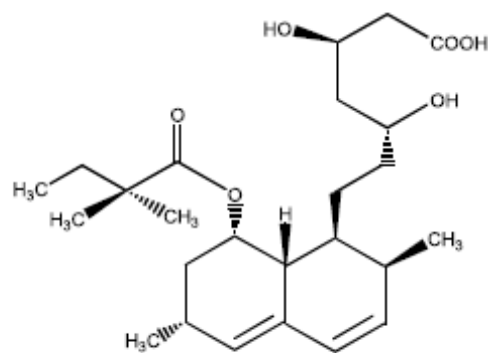
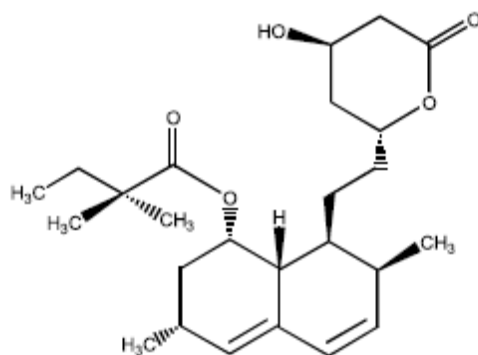
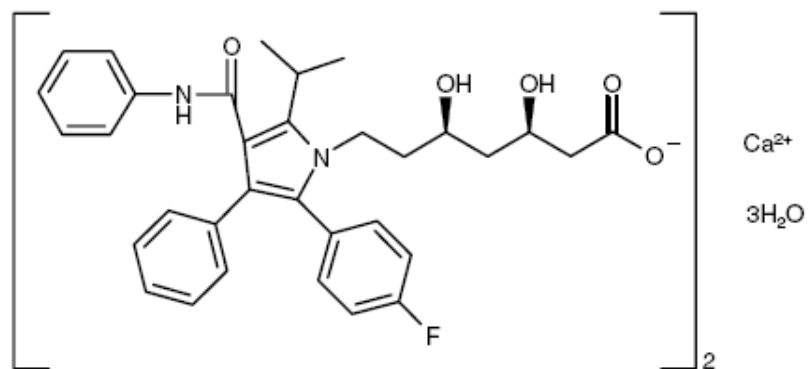


Fig1.13: Chemical structure of Statins: Atorvastatin and Simvastatin are lipophylic while Pravastatin is hydrophilic. Adapted from Pasha et al and van leuven et al^{217,218}.

Statins, by virtue of binding to the active site of the HMG-CoA reductase prevent the substrate-product transition state of the enzyme and reduce the hepatic synthesis of cholesterol²¹⁹. Statins also increase the expression of hepatic LDLc receptors and thus increase the serum clearance of cholesterol. Statins bind to the enzyme with an affinity in the nanomolar range and effectively displace the natural substrate of the enzyme that binds with an affinity in the micromolar range²²⁰. All statins differ in their potency. The newer statins such as Atorvastatin, Rosuvastatin, Pitavastatin, Fluvastatin and Cerivastatin are more potent at reducing cholesterol synthesis when compared to older statins such as Simvastatin, Pravastatin and Lovastatin²²¹. Statins also differ in their lipophylicity and this may influence the ability of lipophilic statins such as Simvastatin and Atorvastatin to have extra-hepatic effects¹⁴. Pravastatin is more hydrophilic because of the hydroxyl moiety.

Statins play a major role in primary and secondary prevention of ischemic heart disease by virtue of reduction in the plasma cholesterol levels. This has been demonstrated by various trials such as the Scandinavian Simvastatin Survival Study (4S), the Cholesterol And Recurrent Events (CARE) study and the West Of Scotland Coronary Prevention (WOSCOPS) study²²²⁻²²⁴.

The advent of the potent statins such as Atorvastatin, heralded numerous trials which suggested significant reduction of cardiovascular events in those with or without coronary artery disease. To name a few, ASCOT-LLA and the 4D studies were carried out in those patients without CAD and the MIRACLE and PROVE IT studies were carried out in those patients with CAD²²⁵⁻²²⁸. It was demonstrated that aggressive lipid lowering with Atorvastatin when compared to Simvastatin resulted in regression of atherosclerosis as seen by a reduction in the carotid artery intima-media thickness²²⁹. Thus there is unequivocal evidence

that aggressive lipid lowering is of definite benefit in primary and secondary prevention of CAD and that this is more effective with the more potent statins such as Atorvastatin.

However it is now becoming increasingly evident that all the beneficial effects of statins may not be related to their ability to lower cholesterol. Indirect evidence from WOSCOPS, ASCOT and the CARE trials, in terms of subgroup analysis, has revealed that the relative risk reduction in cardiovascular mortality and morbidity in individuals in the statin group was still significant despite comparable serum cholesterol levels with the placebo group. This brought to fore the importance of the non lipid lowering actions of statins in cardiovascular protection¹⁴. This will be the subject of discussion in the next section.

1.4.1 PLEIOTROPIC ACTIONS OF STATINS AND CARDIO-PROTECTION

Pleiotropic actions of statins are defined as extrahepatic beneficial effects, that are dependent on mechanisms not related to its ability to lower cholesterol¹⁴. As already explained mevalonic acid levels are reduced as a result of the action of statins on the enzyme. However mevalonic acid is also a precursor for non-cholesterol compounds such as isoprenoids which have numerous actions in cell signalling and cell survival (See figure 1.10). Inhibition of this isoprenoid synthesis has been the focus of much of the pleiotropic actions of statins. Though the pleiotropic actions may vary to some extent depending on the lipophylicity of the specific statin, the actions largely remain similar. Thus my discussion will relate to statins as a group.

The pleiotropic actions of statins in coronary artery disease has been shown to be mediated at the level of vascular endothelial cells, platelets and inflammatory cells and the myocardium itself ¹⁴.

Statins improve endothelial dysfunction by increasing the expression of eNOS and thus increasing NO synthesis and also by reducing endothelin1 production²¹⁴. Simvastatin and Lovastatin have been shown to upregulate eNOS levels in human saphenous vein endothelial cells and this effect was abrogated by mevalonic acid thus suggesting a post transcriptional mechanism²³⁰. Human endothelial cells treated with Mevastatin demonstrate an up-regulation of eNOS and this is abolished by administration of geranylgeranyl pyrophosphate, which is downstream of mevalonate. In the same study inhibition of Rho was associated with an upregulation of eNOS, thus suggesting that statins act by inhibiting Rho in the endothelium²³¹. In a rabbit model of hind limb ischaemia, Simvastatin was shown to rapidly increase eNOS levels in the endothelial cells in a PI3k/AKT dependent manner and this was associated with a decrease in apoptosis²³². Another mechanism of statin induced up-regulation was demonstrated in rat umbilical vein endothelial cells, wherein statin therapy was shown to inhibit caveolin formation (caveoli are negative regulators of eNOS) and also promoted HSP90 (Heat Shock Protein90) induced interaction of AKT with eNOS²³³. Another potential mechanism is related to the antioxidant property of statins. In an in-vivo rat model, Atorvastatin has been shown to improve endothelial function by inhibiting the expression of the p22phox subunit of NAD(P)H oxidase which is a precursor of ROS. It thus reduces oxidative stress. The associated reduction in reactive oxygen species (ROS) will reduce the scavenging of NO and thus result in enhanced availability of NO²³⁴.

Statins have also been shown to inhibit platelet activation and thus have an impact on thrombogenicity. In a normocholesterolemic mouse model, atorvastatin has been shown to up-regulate nitric oxide synthase-3 in platelets and this was associated with decreased platelet activation²³⁵. Statin induced increase in NO availability is also associated with a reduction in p-selectin expression and leukocyte adhesion. This may well play a role in reducing the inflammatory component of reperfusion injury²³⁶.

Statins are protective when given acutely during either of two phases of myocardial ischaemia-reperfusion, the first being prior to ischaemia and the second being at the onset of reperfusion. Experimental models have demonstrated that the beneficial acute effects of statin can be seen to occur when administered from up to 72 hours prior to ischaemia-reperfusion till just prior to ischaemia or reperfusion²³⁷.

Investigation of the potential cardioprotective pleiotropic effects of Statins was initiated in the 1990s by experiments which administered relevant statins pre-ischaemically. One of the early experimental studies pointed to the role of endothelial Nitric Oxide Synthase (eNOS) as being a mediator in statin dependent cardioprotection. In these experiments using the isolated rat heart preparation, Simvastatin was administered 18 hours prior to ischaemia and statin treated hearts had an improvement in the left ventricular developed pressure (LVDP) and left ventricular contractility²³⁸. Similar results in the rat ex vivo preparation were demonstrated when Simvastatin was administered 20 minutes prior to ischaemia. In this study it was shown that the cardioprotective effects are abolished by the administration of the NOS inhibitor, L-NAME (which was also administered 20 minutes prior to ischaemia). This group showed that

this effect is likely to be mediated by the inhibition of isoprenylation of G proteins that inhibit eNOS mRNA²³⁹.

However it still remains unclear as to which is the precise mechanism of statin-induced cardioprotection and various preclinical studies have tried to elucidate this. The following section will review the current available evidence for mechanistic insight into the pleiotropic actions of statins in the setting of myocardial reperfusion injury.

Anti-inflammatory action: Following the initial studies demonstrating the role of eNOS mediated actions of statins, it was also demonstrated that Simvastatin given to diabetic mice for 5 days prior to being subjected to an in vivo model of ischaemia-reperfusion, significantly reduced the infarct size. This was also related to an increase in the availability of NO from eNOS and this was shown to be due to a reduction in the recruitment of PMN cells and thus a reduction in inflammation²⁴⁰.

Activation of RISK pathway: It had also been demonstrated previously that statins promote the differentiation of endothelial progenitor cells by activation of PI3 Kinase/ AKT pathway²⁴¹. It was also shown that statins are responsible for AKT translocation to the membrane domain in endothelial cells and that this was dependent on PI3 kinase and the inhibition of mevalonic acid formation²⁴². Following this Yellon's laboratory demonstrated that acute administration of Atorvastatin at the onset of reperfusion resulted in a reduction in infarct size in a mouse Langendorff model of ischaemia-reperfusion. This was seen to be dependent on PI3 Kinase and AKT activation of eNOS⁸. Similar results were also reported in rat in vivo experiments when Simvastatin was acutely administered during reperfusion²⁴³. Indirect evidence of the involvement of PI3

Kinase/Akt pathway in statins induced cardioprotection was obtained when it was demonstrated that chronic administration of Atorvastatin resulted in loss of cardioprotection and that this was associated with an upregulation of PTEN (Phosphatase and tensin homolog deleted on chromosome ten) which is a known inhibitor of PI3K. However this cardioprotection was recaptured with a high dose of atorvastatin administered pre-ischaemically²⁴⁴. More recently Rosuvastatin administered prior to ischaemia in hypercholesterolemic pigs (in vivo recovery model of acute myocardial infarction) enhanced the levels of PKC, Erk2 and AKT/PKB³⁷⁸.

Statins and Adenosine: Adenosine has been implicated as a mediator of statin-induced cardioprotection. This was first demonstrated in a hypercholesterolemic rabbit in vivo model where Pravastatin restored the ability to pre-condition hearts which was initially blunted by hypercholesterolemia. This was seen to parallel the activation of ecto5'nucleotidase in the ischaemic zone²⁴⁵. In a small study involving 35 hyperlipaemic patients undergoing elective angioplasty for CAD, 3 month treatment with Pravastatin was associated with a reduction in myocardial enzyme release and reduction in ST segment shift peri-procedure and this was abolished by the adenosine blocker aminophylline²⁴⁶.

In vivo experiments in a dog model of ischaemia-reperfusion injury have shown that when statins are administered pre ischaemically there is a dose dependent activation of PI3 Kinase and ecto 5' nucleotidase during reperfusion. Ecto 5' nucleotidase is a source of adenosine²⁴⁷. Adenosine, as already discussed, has an infarct sparing action by virtue of PI3 Kinase dependent activation of mitochondrial K⁺ ATP channels. However this study did not elucidate the exact mechanism of the interaction of statins and the ecto5'nucleotidase. In a further

attempt to unravel the mechanistic interaction of statins and adenosine, immunoblot experiments were carried out on mice hearts in the absence of ischaemia-reperfusion. This study revealed that treatment with statins is associated with activation of A1, A2a and A2b adenosine receptors which in turn phosphorylate ERK1/2 and Akt/eNOS activation²⁴⁸. Further evidence of a potential role for adenosine mediated statin protection comes from rat heart ex vivo experiments carried out using a combination of atorvastatin and dipyridamole and also the effect of caffeinated coffee on statin mediated cardioprotection. Dipyridamole prevents reuptake of adenosine and was shown to work synergistically when administered with atorvastatin for three days prior to ischaemia and this effect was lost on administration of the adenosine receptor blocker aminophylline²⁴⁹. More recently caffeine, which is a known adenosine receptor blocker was shown to inhibit statin-induced cardioprotection when administered pre-ischaemically²⁵⁰. All these studies suggest that statin-induced cardioprotection is mediated by adenosine by an as yet unknown mechanism.

Statins and Isoprenoids: Another suggested mechanism of statin-cardioprotection is the inhibition of mevalonic acid synthesis and its impact on the formation of isoprenoid intermediates. As already discussed statins inhibit the enzyme HMG CoA reductase (which is the rate limiting step in the cholesterol biosynthetic pathway). This enzyme is responsible for the formation of mevalonic acid which is the precursor of both cholesterol and isoprenoids such as farnesyl pyrophosphate and geranylgeranyl pyrophosphate. Isoprenoid synthesis is an independent mechanism from that of the cholesterol biosynthetic pathway¹⁴. The isoprenoids are responsible for activating Rho group

of proteins which have important cardiovascular actions. This synthetic pathway is summarized in **figure 1.14**.

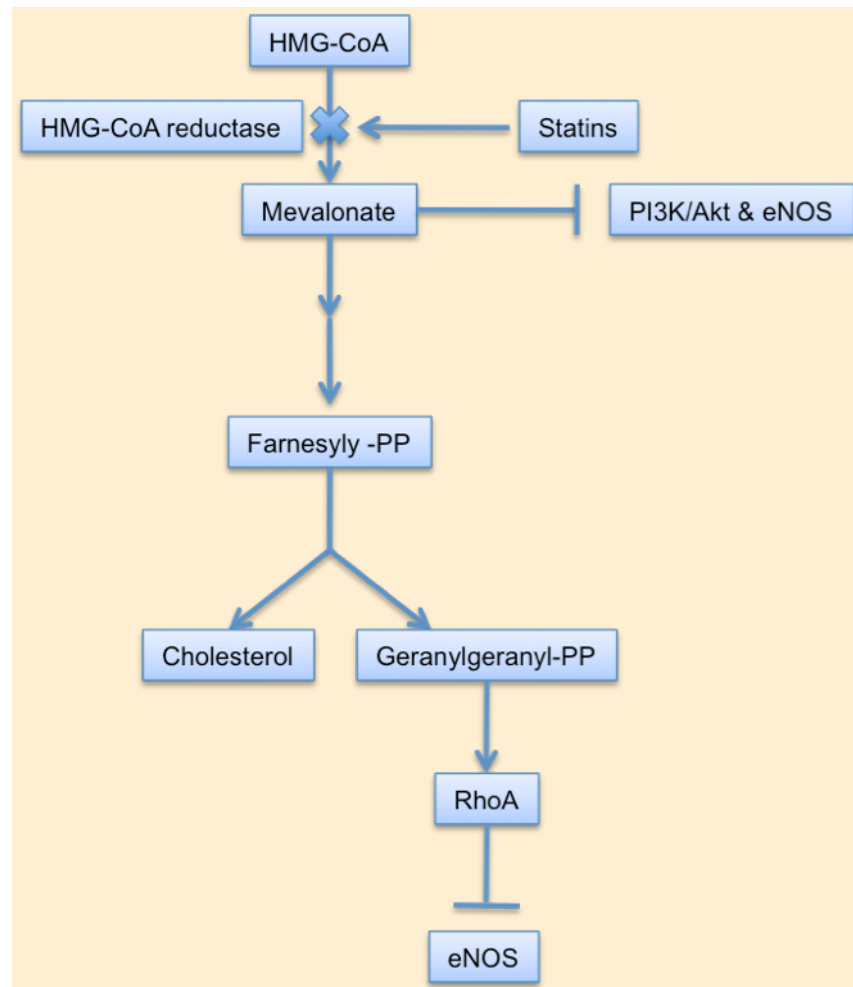


Fig 1.14: Mevalonic acid is the precursor of the isoprenoids Farnesyl pyrophosphate and geranylgeranyl pyrophosphate. These isoprenoids are required for activation of Rho A which is an inhibitor of PI3K and eNOS. Adapted from Liao et al¹⁴.

The Rho group of proteins are GTPases and are members of the Ras superfamily of small GTP binding proteins²⁵¹. Of these the GTPases that have been characterized best are Rho, rac and Cdc42²⁵². These Rho proteins cycle between active GTP bound state which is membrane bound and inactive GDP bound state which remains in the cytosol²⁵³. A key step in Rho activation is its binding to geranylgeraniol which is one of the isoprenoid intermediates of the cholesterol biosynthetic pathway. Rho A further mediates its actions via Rho

associated kinase (ROCK)²⁰¹. ROCK has been shown to mediate hypoxia induced down regulation of eNOS in human pulmonary endothelial cells and this was reversed by the ROCK inhibitor Fasudil²⁵⁴. It has also been demonstrated in vascular smooth muscle cells that ROCK inhibits the activation of PI3 Kinase. Vascular smooth muscle cells were treated with insulin. Insulin was shown to inhibit the action of ROCK. Administration of Wortmanin (the PI3 kinase inhibitor) reversed the action of insulin on the vascular smooth muscle cells but did not have an effect on ROCK inhibition. Thus it was demonstrated that PI3 Kinase inhibition is probably central to ROCK mediated actions²⁵⁵. Acute administration of the ROCK inhibitor Fasudil has been shown to be cardioprotective in an exvivo model of rat heart preparation and this was reversed by the PI3 Kinase inhibitor and the eNOS inhibitor²⁵⁶. This interaction of the Rho A/ ROCK pathway has been shown in **figure 1.15**.

It has been shown that Mevastatin treated mice endothelial cells have an upregulation of eNOS expression which is reversed by treatment with geranylgeranylpyrophosphate and mevalonic acid²³¹. Rosuvastatin administered orally for 48 hours prior to ischemia was also shown to be cardioprotective in an in vivo rat heart model and this was reversed by the administration of mevalonic acid and geranyl geranyl pyrophosphate. Western blot analysis revealed downregulation in active Rho A in the Rosuvastatin group²⁵⁷.

Thus there exists both direct and indirect evidence that statin induced cardiovascular protection could be via the inhibition of Rho A/ ROCK pathway.

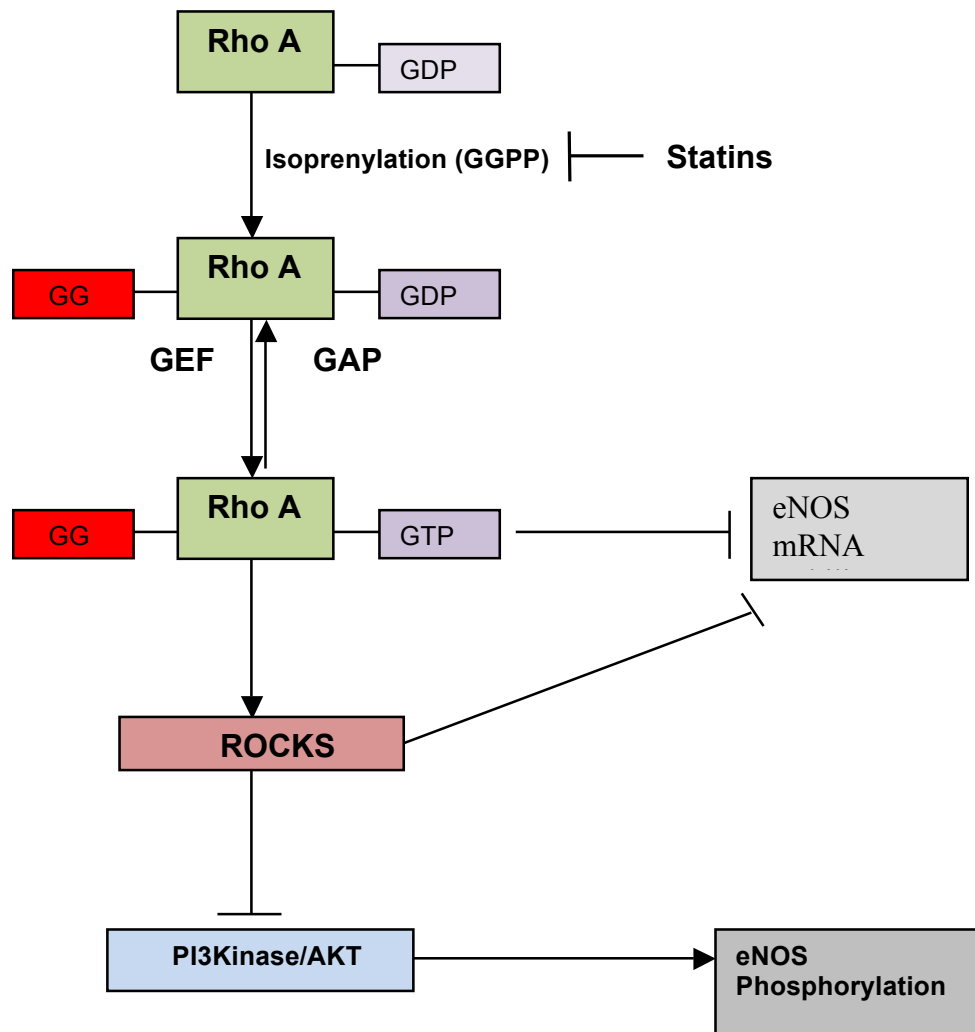


Fig1.15: Statins inhibit the formation of geranylgeranyl pyrophosphate which is required for the isoprenylation of Rho A. Activated Rho A inhibits the mRNA stability of eNOS and also activates Rho Kinases (ROCKs). ROCK is a negative regulator of PI3K/Akt and thus inhibits the phosphorylation of eNOS. GGPP: Geranylgeranyl pyrophosphate GG: Geranylgeraniol, GEF: guanine nucleotide exchange factors and GAP: GTPase activating proteins.

Other mechanisms: Various other potential actions of statins have also been implicated. Cyclooxygenase 2 has been implicated in statin induced cardioprotection by virtue of the upregulation of prostaglandins 6Keto- PGF1 α and PGE2 production²⁵⁸ that is observed in rat ex vivo hearts when rats treated with Atorvastatin for three days prior to ischaemia showed an up regulation of Cyclooxygenase-2 activity and inducible nitric oxide synthase activity (iNOS) both of which have been shown to offer cardioprotection by preconditioning²⁵⁹.

In separate experiments Aspirin administered prior to ischaemia or during reperfusion was shown to reverse the cardioprotection of atorvastatin. Aspirin is a non-selective inhibitor of Cyclooxygenase enzyme²⁶⁰. This mechanism is believed to be downstream of eNOS activation.

Previous experiments conducted in our laboratory have demonstrated that Atorvastatin administered at the onset of reperfusion in Langendorff perfused mice hearts activated other prosurvival kinases such as p44/42 MAPK and p38 MAPK which then activated Heat Shock Protein 27 (HSP27)²⁶¹. It was presumed that HSP27 activation resulted in protection by inactivation pro-apoptotic proteins such as the caspase3, caspase9 and Fas and also by regulating the activity of AKT. However direct causation was not demonstrated in this study.

It has also been shown that Atorvastatin activates peroxisome proliferators activated receptor gamma (PPAR γ) in rat cardiomyocytes and this has been seen to be as a consequence of prostaglandin activation²⁶². In separate experiments the PPAR γ activator Rosiglitazone was shown to be cardioprotective through the RISK pathway²⁶³. The combination of Atorvastatin and Pioglitazone (PPAR γ activator) when administered prior to ischaemia has been shown to be additive in a rat in vivo model. However the downstream effectors appeared to be different. Atorvastatin induced eNOS phosphorylation, iNOS, Cyclooxygenase-2 expression and cytosolic phospholipase expression while Pioglitazone mainly increased the expression of the cytosolic phospholipase³⁷⁹.

More recently Rosuvastatin administered at reperfusion in normocholesterolemic and hypercholesterolemic rabbits (Langendorff perfused

hearts) was shown to reduce infarct size and this was associated with a reduction in the activity of matrix metalloproteinase-2 activity³⁸⁰. Also in a rat in vivo model recently pre-ischaemic administration of Atorvastatin has been shown to attenuate infarct size and also improve left ventricular function following myocardial ischaemia-reperfusion and this was associated with an up-regulation of TNF α and Interleukin-10 mRNA³⁸¹.

These potential mechanisms have been summarized in **table 1.01**.

Mechanism/Mediator	Effector
a. Anti-inflammatory action	↑eNOS, ↓leukocyte adhesion, ↓migration, TNF α and IL-10.
b. RISK Pathway (direct)	↑PI3K/AKT/eNOS, Inhibit MPTP
c. Adenosine	↑PKC/AKT/eNOS, ↑Mito K ATP, Inhibit MPTP
d. Rho A/ROCK Inhibition	↑PI3K/AKT, ↑eNOS phosphorylation & mRNA stability.
e. Cyclooxygenase 2	↑PGE2, ↑iNOS
f. HSP27, MAPK	↑AKT, ↓caspase3 & 9.
g. PPAR γ	↑PI3K/AKT, eNOS, COX-2.
h. ↓ Matrix metalloproteinase-2	↓Cleaving of TroponinI and Myosin light chain protein.

Table 1.01: Pleiotropic actions of Statins in Cardiomyocytes.

There is some evidence to suggest that these pleiotropic actions are important in the clinical setting. The ARMYDA study showed that seven day pre-treatment with atorvastatin 40 mg in patients undergoing elective PCI showed a reduction in peri procedural myocardial injury²⁶⁴. The recent ARMYDA-RECAPTURE study showed that reloading patients on chronic statin therapy who were undergoing elective PCI was associated with a better 30 day outcome²⁶⁵. The recent NAPLES study has also revealed that a single dose of 80 mg of

Atorvastatin prior to PCI was associated with a reduction of periprocedural myocardial infarction²⁶⁶.

1.4.2 ATORVATSTATIN AND CARDIOPROTECTION

Atorvastatin, which is the subject of this thesis, has a molecular weight of 1209.42 kDa²¹⁸. It is a synthetic compound which is lipophylic and is a tissue specific HMG CoA reductase inhibitor with its primary action on the liver²¹⁸. It is insoluble in aqueous solutions at a pH of 4.0, slightly soluble in ethanol and freely soluble in methanol²¹⁸. It is rapidly absorbed when administered orally and has a half life of 20 to 30 hours in humans due to active metabolites²¹⁸.

There is now good preclinical and clinical data to suggest that the pleiotropic actions of statins may be involved in the acute cardioprotection from ischaemia–reperfusion injury and that a better understanding of the underlying mechanism with focus on the reperfusion phase will rationalise therapeutics. The various studies involving Atorvastatin have been summarized in **tables 1.02 and 1.03**.

Thus the focus of the studies presented in this thesis are an attempt to understand the mechanistic actions of atorvastatin on acute cardiovascular protection when administered during reperfusion.

Model	Timing of administration	Result
a. Isolated mouse heart	At reperfusion	↓ infarct size. PI3k/AKT/eNOS activation. (Bell et al) ⁸
b. In vivo Porcine heart	Prior to ischaemia (for 21 days)	↓ infarct size, good wall motion score on ECHO. (Lazar et al) ²⁶⁷
c. In vivo rat heart	Prior to ischaemia (for 3 days)	↓ infarct size, Protection abolished by L-NAME, the Nitric oxide synthase inhibitor. (Birnbaum et al) ²⁶⁸
d. Isolated mouse heart	At reperfusion	↓ infarct size, ↑ PI3K/AKT, ↑ERK1/2 and ↑HSP27. (Efthymiou et al) ²⁶¹
e. Isolated rat heart	Prior to ischaemia (for 14 days)	Protection lost by chronic administration recaptured by acute supplementary dose, ↑ PTEN (PI3K inhibitor) on Chronic treatment (Mensah et al) ²⁴⁴
f. Isolated rat heart	Prior to ischaemia (for 3 days)	↓ infarct size, ↑PGE2 and PGF2 α (Prostaglandins), ↑ eNOS & iNOS. (Birnbaum et al) ²⁵⁸
g. In vivo rat heart	Prior to ischaemia (for 3 days)	↓ infarct size, ↑ iNOS & Cyclooxygenase-2 which is responsible for prostaglandin synthesis. (Atar et al) ²⁵⁹
h. In vivo rat heart	Prior to ischaemia (for 3 days). Co-administered with sildanefil (NOS activator)	Synergistic action with Sildanefil in reducing infarct size. (Rosanio et al) ²⁶⁹
i. Mouse heart Immunoblot	Not Applicable	↑ERK1/2, ↑AKT, ↑ eNOS. All effects abolished by non-specific adenosine receptor antagonist 8-spt. (Merla et al) ²⁴⁸
j. In vivo rat heart	Prior to ischaemia along with Dipyridamole (adenosine reuptake inhibitor)	Synergistic action in infarct size reduction. (Ye et al) ²⁴⁹
k. In vivo rat heart	Prior to ischaemia (for 3 days)	↓ Infarct size. This was abolished by caffeine, a non-specific inhibitor of adenosine receptor. (Ye et al) ²⁵⁰
l. In vivo mouse heart	2 days following ischaemia.	Myeloperoxidase labelled Gadolinium revealed the anti-inflammatory action of Atorvastatin following myocardial infarction. (Nahrendorf et al) ²⁷⁰
m. In vivo rat heart (recovery)	2,7 or 14 days (during ischaemia and reperfusion)	↓ Infarct size associated with up-regulation of TNF α and IL-10 mRNA. (Sun et al) ³⁸¹
n. In vivo rat heart	For 3 days prior to ischaemia	↓ Infarct size, ↑ eNOS phosphorylation, ↑ iNOS, ↑ COX-2 activity and ↑ cytosolic phospholipase activity. (Ye et al.) ³⁷⁹

Table 1.02: Animal studies with Atorvastatin in the setting of myocardial ischaemia-reperfusion.

Trial	No of Patients	Study design	Results
a. ARMYDA ²⁶⁴	153	Randomization to atorvastatin 40 mg or placebo for 7 days prior to elective PCI for stable angina.	Significant reduction in periprocedural myocardial injury (not related to cholesterol lowering).
b. Briguori et al ²⁷¹	451	Randomization between 3 statins (including atorvastatin) and no treatment, for 3 days prior to elective PCI for stable angina	Post procedure ↓CK-MB & ↓Troponin-I were significant in the statin group.
c. NAPLES-II ²⁶⁶	668	Randomization to 80 mg Atorvastatin versus no treatment for 24 hours prior to elective PCI for stable angina	Significant reduction in all cause mortality. Subgroup analysis revealed that the most benefit was seen in patients with a high baseline CRP, suggesting anti-inflammatory actions of statins.
d. ARMYDA-ACS ²⁷²	171	Randomization between 80 mg of Atorvastatin 12 h prior to and a further 40 mg prior to PCI and placebo in patients with Acute Coronary Syndrome.	Composite end points of death, MI and revascularization were significantly lower in the statin group. Periprocedural myocardial injury was also significantly lower.
e. ARMYDA-RECAPTURE ²⁶⁵	383	Randomization between 80 mg Atorvastatin 12 h prior to and 40 mg prior to PCI and placebo in patients on chronic statin therapy, having PCI.	Significant reduction in the incidence of major adverse cardiac events.

Table 1.03: Clinical trials with Atorvastatin in the acute setting.

2. BACKGROUND, AIMS AND HYPOTHESES

2.1. STATIN AND GLUCOCORTICOID RECEPTOR

As discussed in chapter1, Atorvastatin administered at reperfusion has been shown to have a protective effect via the RISK pathway⁸. The precise mechanism of this activation is still unclear. This may well be receptor mediated and it is interesting to note that statins bind with high affinity to the glucocorticoid steroid receptor (GCR) (See **table 2.01**) even at normal therapeutic doses²⁷³.

Drugs	Affinity to GCR
Cortisol	0.5
Prednisolone	3
Atorvastatin	1
Fluvastatin	3
Lovastatin	15
Pravastatin	8
Simvastatin	2
Rosuvastatin	7

Table 2.01: Statins and affinity to GCR. Affinity expressed as bonded energy (K_i) in nmol. Lower the K_i greater the affinity to the receptor. Adapted from Marshall et al²⁷³.

Furthermore a mouse in vivo model of ischaemia–reperfusion demonstrated that the glucocorticoid, Dexamethasone when administered one hour prior to ischaemia was able to protect the heart and this was mediated by nontranscriptional activity of the GCR on the p85 α subunit of the PI3K which subsequently resulted in the activation of eNOS²⁷⁴.

Hypothesis 1 and aim 1: Thus it was hypothesized that Atorvastatin is cardioprotective when administered acutely during reperfusion and this is due to its interaction with the GCR. The aim of this study is to demonstrate a significant reduction in infarct size in an isolated Langendorff perfused rat heart model with the administration of Atorvastatin at reperfusion and to demonstrate

that this would be abrogated by the non-specific glucocorticoid receptor antagonist, RU486.

2.2 STATINS AND ADENOSINE

As already described adenosine is a well studied cardioprotective agent^{166,178,179} and there are numerous preclinical studies to suggest that statin induced cardioprotection may well be due to its effect on adenosine metabolism²⁴⁵⁻²⁵⁰. However all this data has been with statins administered pre-ischaemically and also both in the acute and the chronic setting.

Hypothesis 2 and aim 2: Thus, it was hypothesized that Atorvastatin administered acutely during reperfusion exerted its effect through an adenosinergic mechanism. The aim of this study is to demonstrate infarct size reduction with Atorvastatin administered acutely during reperfusion and prior to ischaemia in a Langendorff perfused rat heart model and to see if this is abrogated by the nonspecific adenosine receptor antagonist 8-Sulpho phenyl theophylline.

2.3 STATINS AND RHO A

Statins reversibly inhibit the enzyme HMG CoA reductase^{213,219}. This results in a reduction of mevalonic acid. Mevalonic acid is a precursor for both cholesterol and isoprenoids such as geranylgeranyl pyrophosphate and farnesyl pyrophosphate, by divergent pathways¹⁴. These isoprenoids are required for the activation of Rho proteins and more specifically Rho A. Rho A further activates ROCK²⁰¹.

Acute administration of ROCK inhibitors in the reperfusion phase has been seen to protect by activating the RISK pathway²⁵⁴. Also, statins administered in the pre-ischaemic phase have shown to protect by virtue of inhibiting the membrane translocation of Rho A²³¹.

Hypothesis 3 and aim 3: Thus, it was hypothesized that Atorvastatin when administered acutely during reperfusion protects the heart by virtue of inhibiting Rho A proteins, which are negative regulators of the RISK pathway. The aim of this study is to demonstrate infarct size reduction with Atorvastatin administered during reperfusion in a Langendorff perfused rat heart model and to show that this is abrogated by the addition of mevalonic acid. We also aim to quantify activated Rho A levels in statin treated hearts by Western blotting.

3. METHODS

3.1 GENERAL

All experiments were carried out in the laboratory premises of Hatter Cardiovascular Institute, University College London. All animal experiments were performed in accordance with the United Kingdom Home Office guidance as per the Animal (Scientific Procedures) Act of 1986, published by Her Majesty's Stationery Office, London. All animals were obtained from the same supplier and were fed a standard pelleted diet with free access to water and were housed under the same conditions.

3.2 CHOICE OF ANIMAL MODEL

The isolated small mammalian heart probably represents the optimal compromise between the quality and quantity of data that can be obtained and its clinical relevance. Large mammalian hearts are more cumbersome to deal with and involve large amounts of perfusate and also the variability in the reproducibility of results²⁷⁵.

My experiments were specifically designed to study pharmacological mechanisms of statin induced cardioprotection; such protection having already been demonstrated in rats by previous work carried out in our lab on Sprague Dawley rats²⁴⁴. We decide to use the rat heart due to its ease of handling when compared to smaller hearts such as those of mice and more specifically ease of recording intraventricular pressure²⁷⁵. Rat hearts are also by far the best characterized small animal model²⁷⁵. Rat hearts are however susceptible to ventricular fibrillation but it is usually easy to terminate these events if they do occur^{275,276}.

Sprague Dawley rats had also been used in our lab to demonstrate mechanisms of preconditioning and postconditioning and also the effect of pharmacological treatments such as Glucagon Like Peptide-1, Erythropoietin, Omapatrilat etc^{173,277-279}. It is thus clear that there is a significant amount of data to suggest that the Sprague Dawley (SD) rat heart is amenable to study the various phenomena associated with ischaemia-reperfusion injury.

It is well known that age matched adult female rats are relatively protected from Ischaemia-Reperfusion injury²⁸⁰. This difference has been variously attributed to be due a muted inflammatory response in the female rat hearts²⁸⁰, an estrogen dependent mechanism of resistance to reperfusion injury²⁸¹ and also due to the increase in p-Akt and phosphorylated Protein Kinase C- ϵ (p-PKC ϵ) levels²⁸². So I chose male SD rats to avoid this confounding factor.

The isolated heart model is a highly reproducible model that can be studied quickly in large numbers and at a low cost^{275,276}. It also allows the measurement of a large number of biological, morphological and pharmacological indices in the absence of various neurohormonal confounding factors associated with the *in vivo* model²⁷⁵. However the major disadvantage of this model is that it is quite removed from the *in vivo* model²⁷⁵ and thus extrapolation to the intact human body may not be always relevant.

This model also allows for easy use of pharmacological agents in the perfusate to study the various effects on reperfusion injury²⁷⁵. It can also remain reasonably stable for many hours^{275,283} and experiments could be carried out in the face of events such as infarction associated pump failure or ventricular arrhythmias which would otherwise jeopardise an *in vivo* experiment²⁷⁵.

We thus chose the isolated rat heart model with Langendorff perfusion using male Sprague Dawley rats for both infarct size measurement and protein assay by Western blot analysis.

3.3 CHEMICALS AND DRUGS

All constituents of the modified Krebs Henseleit buffer were purchased from BDH laboratory supplies (Merck Eurolab, Dorset, England). 2,3,5 triphenyl tetrazolium chloride (ttc) was purchased from Sigma Aldrich company (Dorset, England). RU486, 8-sulpho phenyl theophylline and Mevalonolactone were obtained from Sigma Aldrich Company and Atorvastatin was provided by Pfizer, USA.

3.4 PREPARATION OF HEARTS FOR PERFUSION

3.4.1 ANAESTHESIA AND ANTICOAGULATION

All animals were weighed prior to experimentation. The animals were anaesthetized with intraperitoneal (IP) administration of Sodium Pentobarbital (55mg/kg) and were anticoagulated with 300 IU of Heparin by intraperitoneal administration^{276,284}. The heparin was given as prophylaxis to prevent thrombus formation within coronary vasculature and the ventricles²⁸⁴. All animals weighed between 350 and 500 g.

3.4.2 DISSECTION OF HEARTS

Once the animals were adequately anaesthetized as assessed by the loss of pedal withdrawal reflex to pain, a skin incision was made at the level of the xiphoid sternum. The peritoneum was exposed and the incision was extended to the right and left costal margins and the diaphragm was perforated^{275,276}. The

incision was then continued up the ribs along the right and left axillary lines. The entire anterior chest wall was then reflected upwards to expose the beating heart and the deflated lungs. The heart was then removed by transecting the descending thoracic aorta and was quickly transferred to a plastic boat containing chilled Modified Krebs Henseleit buffer at 4°C^{275,276}. Timing was commenced at the point of cessation of corporeal circulation. The lung, thymic tissue and fatty tissue were dissected and the aorta was slit just distal to the origin of the brachiocephalic artery. These were then mounted on to the cannula attached to the Langendorff system and the ascending aorta was secured to the cannula with 2 × Mersilk sutures and the heart was retrogradely perfused through the ascending aorta. The time from cessation of corporeal circulation to the time of perfusion was less than 3 minutes in order to prevent inadvertent preconditioning as a result of perioperative delay²⁸⁵.



Figure 3.01 : Heart cannulated on to the Langendorff apparatus

3.5 LANGENDORFF PERFUSION

The Langendorff system has been in use since 1895 and is a method of perfusing the isolated heart retrogradely through the aorta under constant pressure or constant flow²⁴⁴. The aorta is cannulated just above the sinus of

valsalva to ensure that the coronary ostia remain patent²⁴⁴. The hydrostatic pressure of the perfusate closes the aortic valve and the coronary bed is perfused. The perfusate then drains into the right atrium and right ventricle through the coronary sinus and is ejected out via the pulmonary artery^{244,275}. Thus the left ventricle essentially remains empty during the entire procedure.

3.5.1 THE LANGENDORFF SYSTEM

The Langendorff system was purchased from ADInstruments Ltd, Oxfordshire, England. This is a self contained system for perfusion of small animal hearts. It is thermostatically controlled to maintain constant temperature of the perfusate and can be used to deliver perfusate at constant pressure or constant flow rate.

The water jacket contains two separate reservoirs for perfusate and these are separated from the water jacket by means of cylindrical glassware. These reservoirs can be independently oxygenated with a mixture of 95% O₂ and 5% CO₂²⁷⁵ (BOC gases, Manchester). The perfusate in the reservoirs is collected by a peristaltic pump that is regulated by a STH pump controller. The perfusate is then circulated to the cannulated rat heart. The perfusate pressure was maintained at a constant level of 70 mm of Hg by means of a physiological pressure transducer connected to a bridge amplifier. This maintains the pressure within an accuracy of ± 1 mm of Hg by means of a negative feedback circuitry.

In order to measure the Left Ventricular End Diastolic Pressure (LVEDP) and also the LV Developed Pressure (LVDP), another physiological pressure transducer attached to a bridge amplifier was connected to an isovolumic latex balloon inserted via the left atrial appendage into the left ventricle. The balloon was inflated to maintain a constant LVEDP of between 5 and 10 mm of Hg. The

heart temperature was monitored by an implantable T-type thermocouple attached to a T type pod. This thermocouple was inserted into the pulmonary artery. The bridge amplifiers and the T type pod were connected to a pod expander which in turn was connected to an 8 channel Powerlab recorder which could run Chart software.

Once the rat heart was cannulated on to the Langendorff system, perfusion was achieved retrogradely by maintaining the perfusate pressure at 70 mm of Hg by means of the pump controller. A perfusion pressure of 70-90 mm of Hg is physiological for invivo rat hearts but the higher end of this range is not entirely suited for the isolated perfused heart²⁸⁶. The flow resistance to crystalloid perfusate is much lesser than that of blood and thus higher pressures can lead to aortic valve incompetence and inadequate perfusion through the coronary bed and also there is a chance of tissue oedema with saline containing perfusate²⁸⁶. As a result a perfusion pressure of between 60 and 70 mm of Hg is considered adequate for the isolated rat heart model. The left atrial appendage was removed and the isovolumic latex balloon was inserted into the left ventricle and inflated to between 5 and 10 mm of Hg in order to maintain a constant LVEDP. The temperature probe was introduced into the pulmonary artery and the perfusate temperature was maintained between 37 and 37.4 °c by means of adjusting the thermostat. The temperature is tightly controlled in this range as hypothermia can reduce the rate of ischaemic damage and infarct size^{287,288} and also result in myocardial dysfunction. Transient heat stress can result in release of heat shock protein which in turn can alter the myocardial response to ischaemia²⁸⁹. The pH of the perfusate was maintained between 7.35 and 7.45 by modifying the gas output. The pH was ascertained by means

of of a blood gas analyser (ABL 705, Copenhagen, Denmark). The CO₂ in the gas is essential to maintain the pH²⁷⁶.

A 3/0 silk suture on a round-bodied surgical needle was passed around the left main coronary artery and the ends of the suture were passed through a plastic tube which was plugged by another conical plastic tube to form a snare. The snare was made by cutting the ends off a plastic pipette. The exvivo rat heart is particularly amenable to regional ischaemia as the left main artery almost exclusively supplies the left ventricle and is visible along the epicardial surface in a groove close to the left atrial appendage²⁸⁶. Regional ischaemia was induced by tightening the snare and this was released to institute reperfusion²⁸⁴.

The flow rate, temperature and developed pressure were monitored, displayed and recorded using a Powerlab system running Chart 4.0 software. Data was displayed using a Personal Desktop Computer. The various experimental protocols will be discussed later.

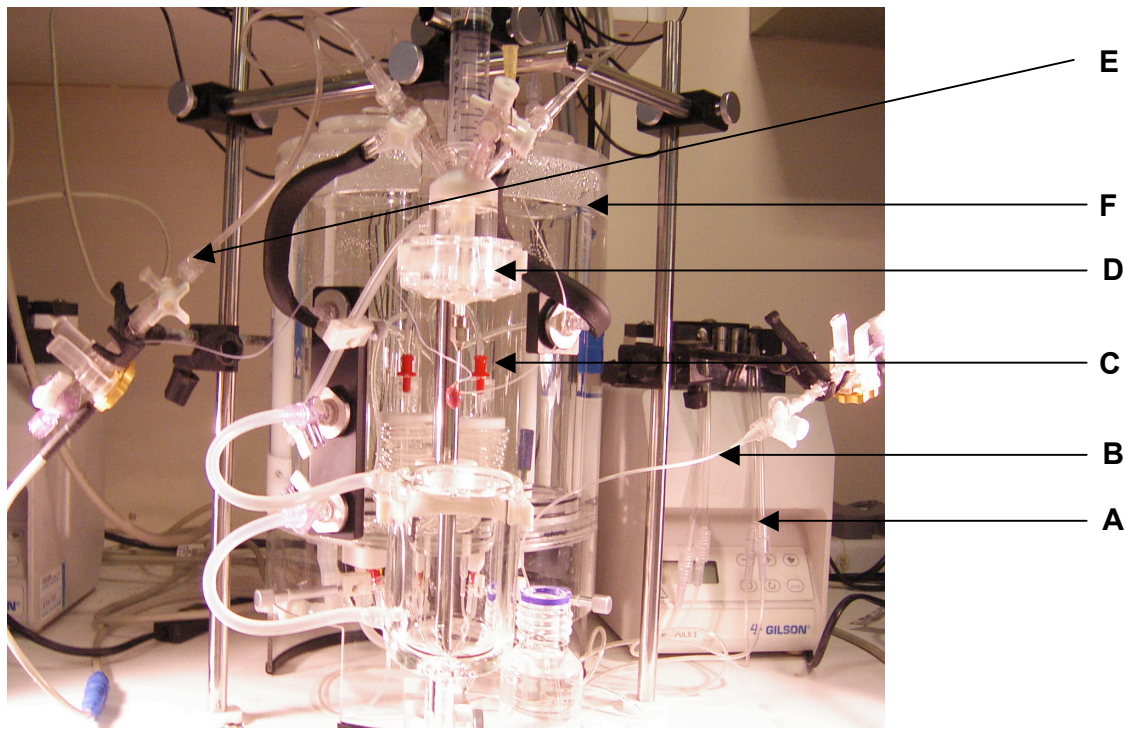


Fig 3.02: Langendorff System. A: Pump Control, B: Pressure transducer for LV, C: Reservoir for buffer/drug, D: Langendorff mount, E: Transducer for perfusion pressure & F: Thermal jacket.

3.5.2 PERFUSION BUFFER

The perfusion buffer was modified Kreb's Henseleit buffer with the following composition (in mM/l): NaCl 118.5, NaHCO₃ 25, KCl 4.8, MgSO₄ 1.2, KH₂PO₄ 1.2, CaCl₂ 1.2 and Glucose 12.0²⁸⁴. Two main kinds of perfusate are typically used for the isolated perfused heart model. These are crystalloid based perfusate like the modified Kreb's Henseleit buffer and blood based perfusate which could be in the form of whole blood pumped from a support animal or washed erythrocytes²⁷⁵. Crystalloid perfusates have ease of preparation, do not require additional perfusion systems and do not have the disadvantage of red cell haemolysis associated with blood based perfusates²⁷⁵. However the crystalloid perfusates have low oxygen carrying capacity with a high pO₂ and thus require high perfusion rates and are also associated with increase incidence of tissue oedema²⁷⁵. However despite this the oxygenation of

crystalloid buffers is sufficient to prevent any deterioration in contractile performance²⁷⁵.

3.5.3 EXCLUSION CRITERIA

The following exclusion criteria were used prospectively:

- (a) Prolonged time to perfusion > 3 minutes. This was to avoid any possibility of inadvertent preconditioning due to perioperative delay as already discussed.
- (b) Coronary flow rate of < 8 ml/min during stabilization.
- (c) Rate Pressure Product (RPP) of < 17,000 at stabilization. This was calculated by multiplying the Heart rate and developed pressure.

The following exclusion criteria were applied retrospectively:

- (a) Hearts with an Infarct size/area at risk % of <12%.
- (b) Hearts with an area at risk/total heart % of < 40% or > 65%.

3.5.4 PARAMETERS MEASURED

3.5.4.1 CORONARY FLOW RATE AND PERFUSION PRESSURE

The perfusion pressure was constant at 70 mm of Hg as already discussed. Thus the coronary flow rate altered based on coronary vascular bed resistance. This was essentially altered by physical ligation of the Left Coronary Artery during ischaemia and releasing this in reperfusion. Some pharmacological agents like calcium channel blockers and nitric oxide donors can also alter this resistance and thus coronary flow rate by virtue of coronary vasodilation²⁸⁴. Verification of occlusion was indicated by reduction in the coronary flow rate by > 30%²⁹⁰.

3.5.4.2 DEVELOPED PRESSURE AND RATE PRESSURE PRODUCT

The developed pressure was measured by inserting an isovolumic balloon into the left ventricle. This also measured the heart rate. The rate pressure product (RPP) was obtained by multiplying the heart rate and developed pressure. The developed pressure and thus the RPP is a measure of ventricular contractility²⁹¹. All hearts with an RPP of <17,000 at the end of stabilization were excluded²⁴⁴. A low RPP would represent non-physiological developed pressure and may well indicate some underlying mechanical dysfunction and thus were excluded prospectively²⁸⁴. It has been seen that the Langendorff model for isolated rat heart is stable at heart rates of up to 450 beats/min. However at higher heart rates the diastolic pressure rises and the developed pressure drops suggesting a decrease in ventricular contractility²⁹¹. All my experiments were well within this upper physiological limit for the heart rate.

3.6 MEASUREMENT OF INFARCT SIZE

3.6.1 TETRAZOLIUM CHLORIDE STAINING

Measurement of infarct size with vital stains is established practice to measure the extent of necrosis following coronary artery occlusion in experimental animals²⁹². One such vital stain is 1,2,3 triphenyltetrazolium chloride (TTC). It is a well established approach for identifying and quantifying irreversible cellular injury. Normal myocardium reduces TTC to a red formazan stain while infarcted tissue remains unstained²⁹³. This is based on the activity of coenzymes, dehydrogenases and NAD/NADP levels in viable tissue when compared to infarct tissue²⁹³. This staining is however dependent on the duration of reperfusion for proper characterization of infarct and in the case of rat heart it has been shown to be a minimum of 60 minutes^{292,294}.

At the end of the experiment protocol, the suture around the left coronary artery was tightened to ligate it and 1.5-2ml of 0.25% evans blue (in saline) was injected through the aortic cannula so as to delineate the area at risk from non-risk area (blue)^{244,292}. The hearts were then frozen in the -20°C freezer for 1-4 hours and the hearts were sliced into 2 mm thick transverse slices (5 slices per heart) from apex to base^{244,284}. These were then incubated in 1% TTC (phosphate buffer) for 12-15 minutes at 37°C²⁸⁴. The infarcted tissue remained pale while the viable tissue in the risk area stained brick red. The slices were then fixed in 10 % formalin for 12-24 hours^{284,292}. The slices were then laid in order in between two glass sheets and scanned onto the PC as a Jpeg image in order to carry out analysis by planimetry.



Figure 3.03: Scanned Image of Heart slices. A: Area at Risk (Pink), B: Non area at risk (Blue), C: Infarcted Tissue (White)

3.6.2 INFARCT SIZE MEASUREMENT

Infarct size analysis was carried out by two methods. Both these methods had comparable results with infarct size analysis and analysis of area at risk (AAR). The early group of experiments were analysed as follows:

After the slices were fixed in formalin, they were laid out in order between two perspex sheets and traced onto acetate sheets. This was subsequently analyzed by computerized planimetry (Summa Sketch iii, Summgraphics, Seymour, CT)²⁴⁴. The acetates were photocopied following magnification by

147%. The planimetry file was opened and calibrated in pixels using a 10×10 mm graph sheet. The outlines were then drawn and the LV cavity was excluded and the remaining area was filled in with yellow to represent the full muscle. The area at risk was then traced and filled with green. The outline of the infarcted tissue was then traced and filled with red. A Microsoft office excel spread sheet was created using simple arithmetic formulae and the following were calculated: muscle volume, Area At Risk (AAR) as a percentage of whole muscle and the infarcted tissue (I) as a percentage of AAR. Results were then presented as I/AAR %.

The second method used with the latter group of the experiments was as follows: In this method the heart slices were laid out in order between perspex sheets and scanned onto a personal computer with a resolution of 600 dpi. The images were stored as Jpeg files and were analysed using the free-share software, Image j Version 5.1. The slices were trimmed by tracing and cutting the unwanted segments including the LV cavity by using the polygon selection function of the software. Then an RGB (Red-Green-Blue) split function was performed. Utilizing the slices in the red channel the slices were filled with red colour and area in pixels was measured to obtain total muscle area. The area at risk was obtained by subtracting the Evan's blue area from the whole muscle using the red channel. The green channel was used to highlight the infarcted tissue and the cumulative infarcted area was obtained by selecting the highlighted infarcted tissue. The results were then represented as Infarct / AAR %. The results were expressed as mean \pm SEM (Standard Error of Mean) and one way ANOVA test (Graphpad Prism software, version 5) with Tukey post test correction was used to test significance. A 'p' value of <0.05 was considered significant.

Any hearts with an AAR of <40% or >65% were excluded from final analysis. Also hearts with an infarct size of <12% or >80% were excluded from the final analysis.

3.7 WESTERN BLOTTING

3.7.1 TISSUE PREPARATION

Rat hearts were isolated and Langendorff perfused as already described. The protocol will be discussed in further sections. At the end of the experiment protocol, the suture around the left coronary artery was tied and the area at risk was delineated using Evan's blue dye. This area was excised, snap frozen using a Wollenberger clamp (pre chilled in liquid nitrogen) and stored at -80°C for further analysis²⁴⁴.

3.7.2 PROTEIN EXTRACTION

The frozen heart samples were added to numbered röhren tubes. Each of these tubes were prefilled with 400 µl of the suspension buffer which consisted of the following: sodium chloride 100 mmol/l, TRIS 10 mmol/l (pH:7.6), ethylene diaminetetraacetic acid 1 mmol/l (pH:8.0), sodium pyrophosphate 2 mmol/l,, sodium fluoride 2 mmol/l, β-glycerophosphate 2 mmol/l, phenyl methyl sulfonyl fluoride 0.1µg/ml and 1 µg/ml each of aprotinin, leupeptin, protease inhibitor and trypsin inhibitor. Each sample was then homogenized with the tubes placed in a beaker of ice. The samples are then centrifuged at 10,000 revs/min and 4°C for 10 minutes.

The supernatant was then divided into two parts: one of 160µl and the other 150 µl. The numbered samples containing 150 µl of the supernatant were

processed separately for assaying activated Rho A levels and this will be discussed later.

Of the 160 μ l of supernatant 10 μ l was used for protein quantification. The remaining supernatant was then mixed with sample buffer containing TRIS 100 mmol/l (pH 6.8), dithiothreitol (DTT) 200 mmol/l, Sodium dodecyl sulphate 2% (SDS), bromophenol blue 0.2% and glycerol 20% and placed in a heating block at 100 °c for 10 minutes. The denatured protein was then stored in the freezer at -80°C.

3.7.3 PROTEIN ESTIMATION

Protein concentrations were calculated by using Bicinchoninic acid (BCA) reagent assay. This assay is based on the biuret reaction of proteins, whereby proteins when placed in an alkaline medium containing Cu^{2+} ions, form a coloured complex between the copper ions and peptide bonds²⁹⁵. BCA is a reagent that can form a 2:1 complex with Cu^{1+} ions and form a stable chromophore with an absorbance maximum at 562 nm²⁹⁵. The relationship between absorbance and protein concentration is linear and forms the basis for protein estimation.

BCA was mixed with copper sulphate solution in a 50:1 ratio. 1 ml of this was then added to duplicate samples of known concentrations of Bovine Serum Albumin (BSA) in suspension buffer and also duplicate samples of the proteins to be estimated. A standard absorption curve was generated by using the BSA and measuring the absorbance with a spectrophotometer. The extracted protein absorption was then calculated and compared with the standard curve. A mean value of protein concentration was then calculated to enable equal loading of the polyacrylamide gel.

3.7.4 SAMPLE PROCESSING FOR ACTIVATED RHO A

Activated Rho A is membrane bound and thus tissue samples need to be processed to release the membrane bound activated Rho A prior to estimation by gel electrophoresis. Thus Rho A activation was determined by using an affinity precipitation assay incorporating the Rho-binding domain (RBD) of rhotekin, which binds only to the active GTP bound form of Rho A²⁹⁶.

As already explained the supernatant obtained from homogenization and centrifugation was divided into two samples of 160 μ l and 150 μ l respectively. The sample with 150 μ l of supernatant was processed as follows to release activated Rho A: To each sample was added 20 μ g of agarose conjugated Rhotekin-RBD (30 μ l/sample). This slurry was obtained from Upstate cell signalling solutions, Lake Placid, NY. The samples were then incubated at 4°C for 45 minutes and then centrifuged at 4°C for 3 minutes at 15,000 revs/minute. The supernatant was discarded as the Rhotekin-RBD slurry would bind to the active Rho-A and settle to the bottom of the Eppendorff. The samples were then washed 3 times with the washing buffer (0.5 M/l NaCl, 1% Tx100, 10 mM of Magnesium chloride, 2ml of Sodium pyrophosphate, 400 μ l of sodium fluoride, 500 μ l of PMSF/AESBSF and 2.5 tablets of protease inhibitor).

To this was then added 150 μ l of suspension buffer (Section 3.7.2) and then 150 μ l of the sample buffer (Section 3.7.2), to obtain a final volume of 300 μ l. The samples were then heated at 95-100 °C for 5 minutes to release the Rho A from the beads. The samples were then loaded on to the polyacrylamide gels at the same concentration as that obtained from BCA assay of the corresponding sample for total Rho A.

3.7.5 POLYACRYLAMIDE GEL ELECTROPHORESIS

3.7.5.1 PRINCIPLES OF SODIUM DODECYL SULPHATE (SDS) POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE) AND WESTERN BLOTTING

SDS-PAGE is the most widely used method for qualitatively analyzing proteins²⁹⁷. The proteins separate on the polyacrylamide gel based on their relative molecular mass (size) and also native charge²⁹⁷. This method works on the principle that denatured proteins loaded on to a gel base will migrate when a current is passed through the gel and this migration and separation is dependent on (a) pore size of the gel (b) native charge of the protein and (c) the quaternary structure of the protein²⁹⁷.

However DTT in the sample buffer reduces any disulphide bridges holding the tertiary structure of the protein together²⁹⁸. Thus the denatured protein opens up into a rod shaped structure and the native charge of the protein is swamped by the SDS²⁹⁸. The negatively charged protein –SDS complexes move towards the anode when the current is passed. As their charge per unit length is the same they move in the running gel with the same mobility²⁹⁷. The proteins in the running gel separate by virtue of the molecular sieving properties of the running gel whereby the smaller proteins are able to pass through the pores while larger proteins are retarded by frictional resistance²⁹⁷. A stacking gel is used to concentrate the protein sample so as to provide sharper bands in the running gel and this works on the principle of isotachopheresis²⁹⁷. The principle of isotachopheresis works on the basis that negatively charged glycinate particles in the buffer have lesser electrophoretic mobility than the SDS-protein complexes which in turn are less mobile than the chloride ions. Field strength is

inversely proportional to conductivity which in turn is directly proportional to concentration²⁹⁷. Thus the SDS-protein complexes are at smaller concentration and form a tight band in the stacking gel.

Once the proteins are separated in the gel, they are transferred to a nitrocellulose sheet. The epitopes of the proteins are now accessible to immunoblotting.

3.7.5.2 GEL PREPARATION

The running gel (12.5% Acrylamide gel) was prepared by mixing 12 ml of deionised water, 15 ml of 30% Acrylamide and 9 ml of Running gel base (1.5 M of TRIS and 0.4% SDS mixed in deionised water, pH 8.8). To this was added 40 µl of NNN-tetraethylethylenediamine (TEMED) and 200 µl of Ammonium persulphate (APS) (10%). The TEMED quickly polymerizes the acrylamide to form a gel. The running gel was then rapidly transferred to the vertical plate assembly and was then topped up with a 50:50 dilution of deionised water and isopropanol. This was allowed to set for 30 min. The stacking gel (5% Acrylamide gel) was then prepared by mixing 2 ml of 30 % acrylamide, 8% bromophenol blue, 3ml of stacking gel base (0.5 M TRIS and 0.4 % SDS in deionised water, pH of 6.8). This was then mixed with 24 µl of TEMED and 120 µl of 10 % APS. The mixture of deionised water and isopropanol was emptied from the vertical glass plates and the combs were removed. The stacking gel was then added on top of the running gel and allowed to set for 10 minutes. Once the stacking gel had set the combs were removed to enable the wells to fill up with running buffer.

3.7.5.3 GEL ELECTROPHORESIS

Precision Plus Protein Standard marker was added to the first well (15 μ l) and appropriate volumes (as calculated by protein curve) of samples were added into corresponding wells. The rig was then placed in the tank and the tank was filled with running buffer. The gel was allowed to run for 2 hours at 200 V, until the marker has spread out sufficiently at the level of the target protein.

3.7.5.4 PROTEIN TRANSFER

Once the gel was satisfactorily run, the gel was taken out and the stacking gel was removed. The gel was then transferred to a Whatman paper placed on the electrophoretic cassette and this was done while the cassette was immersed in transfer buffer which was composed of 20% methanol in deionized water and 10x transfer buffer (150 mM of Glycine and 20 mM of TRIS base)²⁹⁹. An equivalent size Hybond ECL nitrocellulose membrane was then cut and placed on the gel while in the transfer buffer and the top right corner was snipped to identify well number one²⁹⁹. Another Whatman paper soaked in transfer buffer was then placed on top of this nitrocellulose membrane and the cassette was closed and mounted in a tank filled with transfer buffer. Transfer was allowed to occur overnight at 0.1 Amps. Satisfactory transfer was confirmed using Ponceau red staining.

3.7.6 IMMUNOBLOTTING

The membranes were washed on a mechanical shaker with washing buffer (50 ml of 10X TBS, 450 ml of deionized water and 0.5 ml of Tween 20. The membranes were then covered with blocking buffer (washing buffer + 5% skimmed milk) for one hour. The blocking buffer was used to reduce non-

specific binding sites and also to promote renaturation of antigenic sites³⁰⁰. Following blocking, the membranes were washed for 3 × 5 minute periods in washing buffer.

3.7.6.1 PRIMARY ANTIBODY

The washed membranes were incubated with primary antibody (concentration of 1:200 dissolved in 5% Bovine Serum Albumin). The membranes were incubated overnight in the cold room so as to ensure good quality blots.

The primary antibody, Rho A (26C4), was obtained from Santa Cruz Biotechnology. This antibody is a mouse monoclonal antibody raised against an epitope of Rho A of human origin. In order to obtain an internal control, the blots were probed for β -actin³⁰⁰. The primary antibody for β -actin was mouse monoclonal anti β -actin antibody which was purchased from Sigma-aldrich chemicals. The membranes were stripped with 0.2 M Sodium hydroxide and stripping buffer before probing for β -actin.

3.7.6.2 SECONDARY ANTIBODY

The membranes were rinsed with TBS and the specific secondary antibody was added at a concentration of 1:1000. This secondary antibody is usually coupled with a Horse Radish Peroxidase (HRP). Membranes were then developed using an enhanced chemiluminescence technique (ECL Western blotting detection system, Amersham). Bands on the membranes were visualized using autoradiography onto Kodak films (Herts, England).

3.7.7 QUANTIFICATION AND STATISTICAL ANALYSIS

The films were scanned and saved as jpeg images. The blots were then analysed with ImageJ software and relative densitometry values were calculated and converted as a ratio of the β -actin density. Values were expressed as mean \pm SEM. Statistical significance was measured using one way ANOVA test (Graphpad Prism software, version 5.0) with Tukey post test correction and a 'p' value of <0.05 was considered significant.

4. MODEL CHARACTERIZATION AND VALIDATION

4.1 ISCHAEMIA/REPERFUSION PROTOCOL

The ischaemia/reperfusion protocol that involves transient ligation of the coronary artery followed by reperfusion, is the choice of model for studying therapies and outcomes from ischaemia-reperfusion³⁰¹.

An optimum duration of ischaemia and reperfusion was arrived upon based on available literature and the protocol followed in our lab. Regional ischaemia was induced by ligating the left coronary artery with a suture and snare and reperfusion was reinstated by releasing the snare³⁰².

Rat hearts subjected to brief periods of ischaemia (30 to 35 minutes) regained contractile activity after institution of reperfusion. However if the period of ischaemia was 45-55 minutes the hearts do not regain their contractility³⁰³. It has also been shown that the time taken for repletion of myocardial ATP during reperfusion is dependent on the duration of ischaemia³⁰⁴. In addition it has been shown that a minimum of 30 minutes of ischaemia is required to demonstrate complete loss of glycogen stores in rat hearts³⁰⁵. The duration of ischaemia also has an effect on the formation of well defined infarcted tissue which is subsequently delineated by the use of TTC. TTC is able to detect infarcted tissue with ischaemic episodes lasting for 30 minutes or more³⁰⁶. A number of early studies have also shown an increase in adrenergic activity in the ischaemic myocardium and thus the efflux of H^{3+} tagged Nor-adrenaline has been used as a surrogate marker of the degree of ischaemia. It has been demonstrated that the efflux peaks at reperfusion after 30 minutes of ischaemia in rat hearts when glucose has been used in the perfusate, indirectly showing the adequacy of ischaemia³⁰⁷. Furthermore our lab has successfully used a

model involving 35 minutes of regional ischaemia²⁸⁴. Thus, in order to balance the reproducibility of infarction with the requirement for adequate staining with TTC, the duration of ischaemia was set at 35 minutes after a 40 minute period of stabilization.

Reperfusion following ischaemia is essential for myocardial salvage which is manifested as a reduction in infarct size. This has been demonstrated in rat hearts, whereby the size of infarction was 60 % less in those hearts which had been reperfused when compared to those with permanent ischaemia. This infarct sparing was perceptible within the first hour of reperfusion³⁰⁸. The duration of reperfusion is also important as it is required for the washout of dehydrogenases which mediate the TTC staining. It has been shown in rat hearts (in vivo) that a period of 60 minutes or more of reperfusion is essential to obtain homogenous pale white delineation of the infarcted myocardium and any period lesser than 60 minutes results in patchy delineation of infarct tissue which makes it difficult to accurately assess infarct to risk ratio²⁹². However the period of ischaemia in the in vivo experiments was at least 90 minutes. As we used a rat Langendorff method with a 35 minute duration of ischaemia, we chose a reperfusion period of 120 minutes which has been shown to produce reproducible results for infarct analysis in our lab²⁸⁴. However recently it has been demonstrated that 60 minutes of reperfusion is also sufficient to assess infarct size in rat heart Langendorff model²⁹⁴.

Atorvastatin and various inhibitors were administered during the last 5 minutes of ischaemia and for the first 15 minutes of reperfusion except in the experiments involving pre-ischaemic administration of Atorvastatin. This was done so as to allow the drug to diffuse into the cells and activate/inactivate any potential targets, by the time reperfusion is established. This is because any

pharmacological manipulation to attenuate ischaemia-reperfusion injury needs to be present in the first few minutes of reperfusion as this is the critical window of cardioprotection^{127,294}. This has been demonstrated in isolated perfused rat hearts whereby administration of sangliferin A (inhibitor of MPTP) at the beginning of reperfusion attenuates infarct size but not when administered after the first 15 minutes of reperfusion¹²⁷. Thus in my experiments Atorvastatin was perfused towards the end of ischaemia to ensure its availability at the onset of reperfusion.

In the case of pre-ischaemic administration of Atorvastatin, the hearts were perfused with the drug for a total duration of 20 minutes during stabilization, followed by a 10 minute washout period prior to ischaemia.

4.2 ISCHAEMIC PRECONDITIONING

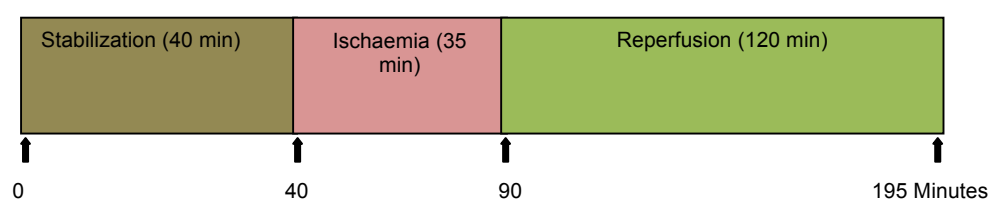
In order to validate my model, I compared the infarct sizes from a standard ischaemia –reperfusion protocol with that of a preconditioning protocol. The results were compared with previous work that used a similar model.

Ischaemic preconditioning of the myocardium is a phenomenon whereby brief periods of sublethal ischaemia would delay the onset of necrosis during subsequent lethal ischaemic insult²¹. In cardiovascular research this translates as a significant and highly reproducible reduction in infarct size. The preconditioning protocol consisted of two cycles of 5 minute periods of global ischaemia interspersed with a 10 minute period of reperfusion, prior to regional ischaemia. The duration of the regional ischaemia and reperfusion period was the same as that of the standard ischaemia-reperfusion protocol. For the ischaemia–reperfusion protocol we used a stabilization duration of 40 minutes followed by a 35 minute period of regional ischaemia and a 120 minute period

of reperfusion. A total of 18 hearts were used. 3 hearts were excluded for technical reasons and data from 5 hearts was excluded either due to very low infarct size (<12%) or due to low risk zone (<40%).

Hearts were randomly assigned to the ischaemia-reperfusion protocol or to the preconditioning protocol as per **figure 4.01**.

A. Ischaemia-reperfusion Protocol



B. Preconditioning Protocol

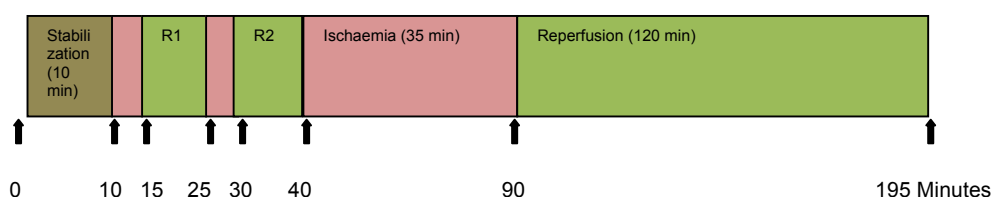


Figure 4.01: Protocols for standardization of the model. Legend: P1 and P2: Preconditioning cycles 1 & 2 of global ischaemia (5 minutes each). R1 & R2: Reperfusion 1 & 2 after P1 & 2 respectively (10 min each).

At the end of the experiments the hearts were stained with TTC and infarct size measured. Data were represented as % Infarct/AAR and a 'p' value of <0.05 was considered significant. Tests of significance were performed using unpaired t-test. Although the baseline morphometrics were not used to validate the model, there was no significant difference in any of the baseline parameters between the control group and the preconditioned group. The results are presented in **table 4.01, 4.02 and 4.03 & figure 4.02**.

Baseline Characteristics

Groups	Number	Body weight
Control	6	445±21
Preconditioning	4	361±20 *

Table 4.01: Body weight (gms ± SEM). * 'p' value of < 0.05 when compared to control.

Groups	S10	S30/P2	I5	I30	R5	R30	R120
Control	14±1.3	13.5±1.4	8±1	7.3±0.8	14.5±1	13±0.7	8.7±1
Preconditioned	14±1.3	14.5±2.2	8±1.3	9±1.3	15.6±1.5	13.5±1.2	6.8±0.8

Table 4.02: Coronary flow rate (ml/min ± SEM). S: Stabilization, I: Ischaemia, R: Reperfusion, P2: Preconditioned cycle 2.

Groups	S10	S30/P2	I5	I30	R5	R30	R120
Control	27378± 3832	25307± 2341	9937± 986	16470± 1195	26336± 1525	25009± 2407	14797± 3312
Preconditioned	44663± 6263*	31404± 3941	18943± 3941	25148± 4506	34062± 4597	28957± 2289	19222± 4951

Table 4.03: Rate Pressure Product. S: Stabilization, I: Ischaemia, R: Reperfusion, P2: Preconditioned cycle 2.

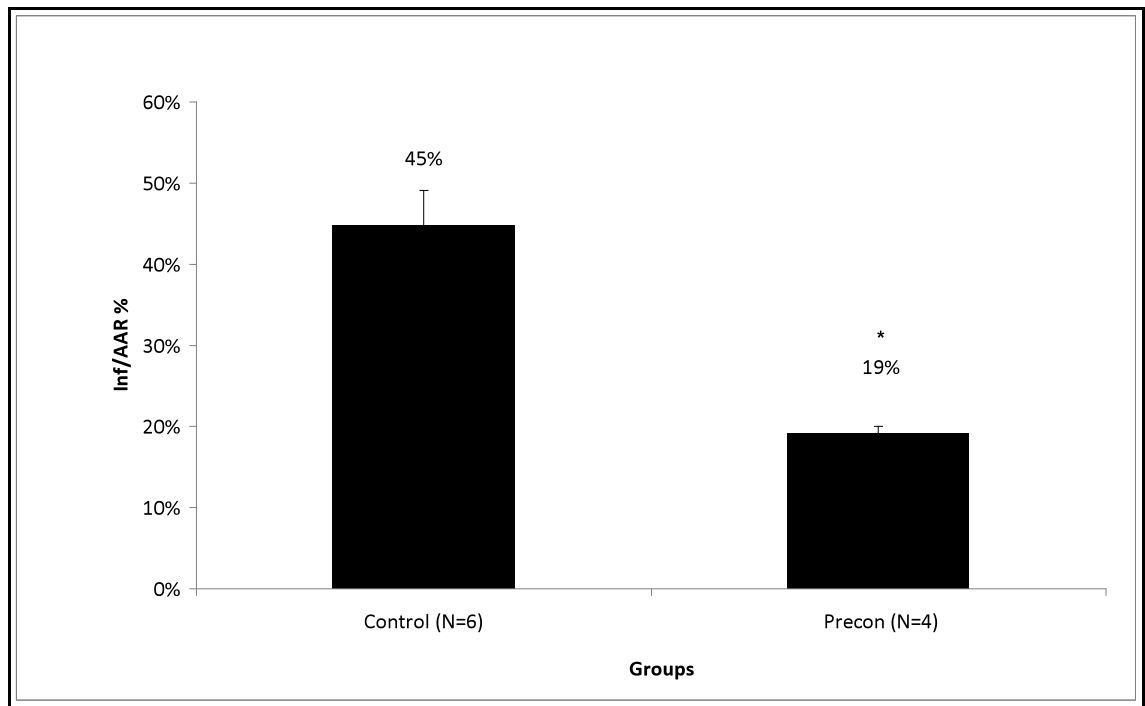


Fig 4.02 : The infarct size as a % of Area At Risk for the control (Ischaemia-reperfusion group) was 45% \pm 4% and the infarct size in the preconditioned group was 19 % \pm 1%. The infarct size reduction was significant with a p value of 0.003.

The infarct size in the control group was 45 \pm 4% and in the preconditioned group was 19 \pm 1%. The infarct size reduction with preconditioning was significant (P value of < 0.01). These results were consistent with previous data obtained in our lab²⁷³. Thus I was able to successfully demonstrate that the model is suitable to obtain reproducible results to demonstrate myocardial protection.

4.3 ATORVASTATIN INDUCED CARDIOPROTECTION IN THE SETTING OF ISCHAEMIA-REPERFUSION

As part of the process to validate the model in my hands, I also verified that Atorvastatin when administered acutely during reperfusion was able to reduce the infarct size in Langendorff perfused rat hearts.

Di Napoli et al. first demonstrated that Simvastatin perfused pre-ischaemically in Langendorff perfused rat hearts attenuated reperfusion injury by a nitric oxide dependent mechanism²³⁹. Bell et al demonstrated in an ex vivo model of mouse heart that Atorvastatin when acutely administered during reperfusion reduced the infarct size and this was by upregulation of the RISK pathway⁸. By using Fluvastatin administered intravenously prior to ischaemia, Teifenbacher et al (rat in vivo model), demonstrated a reduction in infarct size³⁰⁹. Wolfrum et al also demonstrated in a rat in vivo model that acute administration of Simvastatin during reperfusion injury results in a reduction in infarct size by targeting the RISK pathway²⁴³.

In order to demonstrate cardioprotection with acute administration of Atorvastatin at reperfusion, the hearts were divided into 3 groups as follows:

(a) Control Hearts: Which underwent a standard ischaemia-reperfusion protocol (**Protocol A in figure 4.01**).

(b) Vehicle Control (Methanol): Atorvastatin is only soluble in methanol. Thus to demonstrate that the vehicle did not contribute to the cardioprotection, the group consisted of hearts which were perfused with methanol for the last 5 minutes of ischaemia and the first 15 minutes of reperfusion. This was used at a concentration of 0.1%, and this concentration was not associated with any significant protection in an isolated perfused mouse heart⁸ (**Figure 4.03**).

(c) Atorvastatin group: In this group the hearts were perfused with Atorvastatin for the last 5 minutes of ischaemia and the first 15 minutes of reperfusion. Atorvastatin was used at a dose of 50 $\mu\text{mol/l}$ ⁸ (**Figure 4.03**).

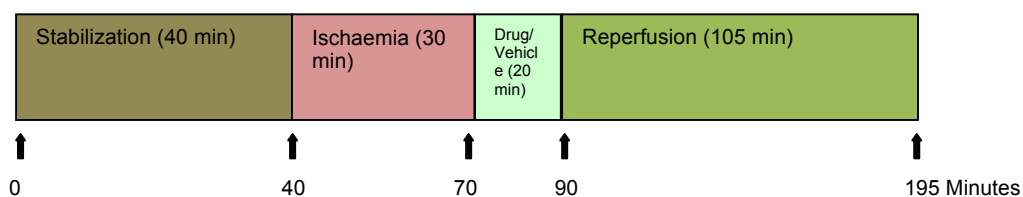


Fig 4.03: Protocol for administration of Vehicle (Methanol at a concentration of 0.1%) and Drug (Atorvastatin at a dose of 50 μ mol/L).

At the end of the protocol the hearts were analysed for infarct size as already described and expressed as Infarct/AAR % and tested for significance by one way ANOVA testing (Graphpad prism software, version 5.0) and Tukey post test comparison and a p value<0.05 was considered significant.

Baseline Characteristics

Groups	Number	Body weight
Control	8	424 \pm 21
Methanol (Vehicle)	6	461 \pm 18
Atorvastatin	7	434 \pm 15

Table 4.04: Body weight (gms \pm SEM).

Groups	S10	S30	I5	I30	R5	R30	R120
Control	14 \pm 1	13 \pm 1	8 \pm 0.8	7 \pm 0.6	14 \pm 0.8	12 \pm 0.7	8 \pm 0.9
Methanol	16 \pm 1	16 \pm 1	8 \pm 0.9	8 \pm 1	15 \pm 0.7	12 \pm 0.9	6 \pm 0.6
Atorvastatin	16 \pm 0.8	14 \pm 0.3	7 \pm 0.3	6 \pm 0.4	15 \pm 1	11 \pm 1	5 \pm 0.3*

Table 4.05: Coronary flow rate (ml/min \pm SEM). S: Stabilization, I: Ischaemia, R: Reperfusion.* p<0.05 when compared to control.

Groups	S10	S30	I5	I30	R5	R30	R120
Control	31077 ±3918	27563 ±3232	11369 ±2159	18517 ±1796	27649 ±1796	26330 ±2169	16313 ±2782
Methanol	30097 ±5431	30020 ±4149	13807 ±2782	21222 ±3190	26025 ±2532	24290 ±3546	14786 ±4390
Atorvastatin	35863 ±4929	32363 ±2946	15424 ±2406	16170 ±2438	23703 ±2672	24027 ±2409	11411 ±1696

Table 4.06: Rate Pressure Product. S: Stabilization, I: Ischaemia, R: Reperfusion,

There were no significant differences in the baseline haemodynamic data for all the groups (Table 4.04, 4.05 and 4.06). The mean infarct size with the control group was 48±5%, the methanol group was 49±4% and the Atorvastatin group was 26±5% (**figure 4.04**). This reduction in infarct size with Atorvastatin was significant when compared to both the control groups and the methanol groups (p value of <0.05).

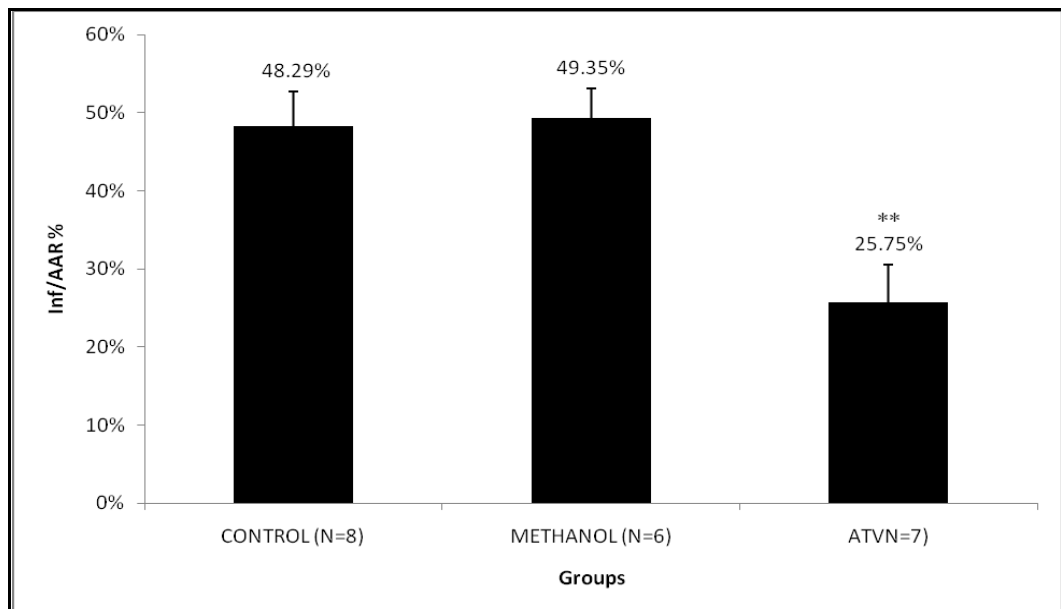


Fig 4.04: Infarct size reduction with Atorvastatin was significant with a p value of <0.05, when compared with both control and vehicle. There was no significant difference between the control hearts and the vehicle perfused hearts.

These results thus demonstrate that Atorvastatin, administered acutely during reperfusion, is able to protect the Langendorff perfused rat heart. This is comparable to the results of previous work in a Langendorff perfused mouse model^{8,261}. It is thus apparent that the infarct sparing action of statins must be as a direct result of its action on the myocardium and independent of its activity on platelets and inflammatory cells (as the perfusate lacks either of these) and also independent of its cholesterol lowering action, as any of these effects require chronic administration of statins over a period of hours to days rather than the 20 minutes used here. We thus set out to explore the possible mechanisms/receptor targets of statin induced cardioprotection in the acute setting.

5. ATORVASTATIN AND THE STEROID RECEPTOR

5.1 AIMS AND HYPOTHESIS

Statins acutely administered at reperfusion have been shown to exert a cardioprotective effect in isolated perfused mouse hearts and in vivo rat hearts^{8,243}. This effect seems to be due to the activation of the pro-survival pathway called the RISK pathway^{8,243}. Corticosteroids such as dexamethasone have also been shown to protect the heart when administered acutely during reperfusion and this effect is exerted via the RISK pathway^{274,310}. This action of the steroids has been shown to be a non-transcriptional action of the glucocorticoid receptor (GCR)^{274,310}. Statins have also been shown to bind to the glucocorticoid receptor at therapeutic doses. Thus we investigated the hypothesis that Atorvastatin protects the rat ex vivo heart preparation when administered acutely during reperfusion and that this acute cardioprotective action is mediated via the GCR.

5.2 METHODS

As described in chapter 3 and 4 (methods & model characterization), Langendorff perfused rat hearts were subject to 35 minutes of regional ischaemia followed by 120 minutes of reperfusion. This protocol was preceded by a 40 minute period of stabilization. At the end of the experimental protocol the hearts were analysed for infarct size as a percentage of the Area at Risk (AAR) using planimetry and data was represented as mean \pm SEM. The data was assessed for statistical significance using one way ANOVA test with a p value of <0.05 considered significant. 52 hearts were used of which one heart was lost due to technical problems. Data from 13 hearts were not included due to significant precipitation of Atorvastatin in the Langendorff system, large risk

zone and small infarct size (i.e., outside the predefined exclusion criteria). This problem with precipitation of the drug was overcome by heating the buffer at 37°C while dissolving the drug and also by minimizing the amount of gas required to maintain the pH in physiological range (Forceful gassing was associated with greater degree of precipitation).

The hearts were randomly assigned to the following groups based upon the drugs administered during reperfusion (**Figure 5.01**):

(a) *Controls*: Standard ischaemia –reperfusion protocol. (Protocol A in figure 4.01).

(b) *Ethanol* as vehicle for the GCR blocker RU486. Ethanol was used at a concentration of 0.005%.

(c) *Methanol* (vehicle for atorvastatin) was used at a concentration of 0.1%. This was used as the control group.

(d) *RU486 (Mifepristone)*, which is the GCR blocker, at a dose of 1µmol/l. RU486 was shown to block the glucocorticoid receptor activity at this dose when used to treat primary adult rat cardiomyocytes³¹¹.

(e) *Atorvastatin (ATV)* at a dose of 50 µmol/l. Atorvastatin was dissolved in methanol at a dilution of 5 mg per 100 µl of methanol. This dose was demonstrated to be cardioprotective in a mouse ex vivo model⁸.

(f) Combination of *Atorvastatin and RU486* at the same concentration as stated above.

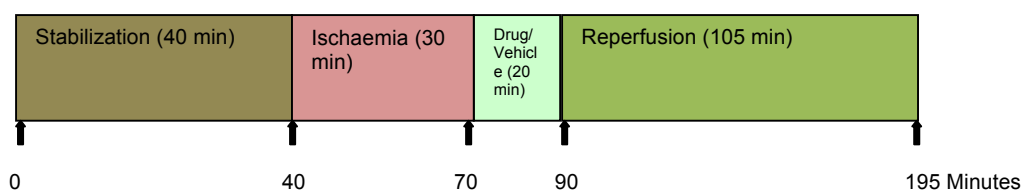


Figure 5.01: Protocols; Vehicle: Ethanol 0.005% or Methanol 0.1%. Drug: Atorvastatin, RU486 and ATV+ RU486

5.3 RESULTS

No significant difference existed between the various groups during stabilization with respect to coronary flow rate, developed pressure and rate pressure product with the exception of the ethanol group (**tables 5.01, 5.02 and 5.03**). These hearts had a significantly better flow rate during stabilization when compared to the control group. However all drug treatments and vehicle treatments occurred acutely during reperfusion and thus this difference would not be influenced by any drug treatment and the noticed difference is probably purely by chance.

Baseline Characteristics

Groups	Number	Body Weight (gms±SEM)
Control	8	424±21
Methanol	7	452±17
Ethanol	10	443±17
Atorvastatin (ATV)	7	433±15
RU486	8	424±12
ATV+RU486	6	411±18

Table 5.01: Body Weight (gms±SEM).

Groups	S10	S30	I5	I30	R5	R30	R120
Control	14±1	13±1	8±1	8±1	14±1	12±1	8±1
Methanol	16±1	16±1	8±1	8±1	14±1	11±1	6±1
Ethanol	18±1*	15±0.3	7±1	7±0.4	15±0.3	10±1	6±0.4
ATV	16±0.4	14±1	7±0.3	6±0.4	14±1	11±1	5±0.3
RU486	17±1	15±0.3	8±1	7±1	15±1	11±1	7±1
ATV+RU486	16±1	15±1	7±1	8±1	14±1	10±1	6±1

Table 5.02: Coronary Flow rate (ml/min ± SEM). S-stabilization, I-Ischaemia & R-Reperfusion. * p<0.05 when compared to control.

Groups	S10	S30	I5	I30	R5	R30	R120
Control	31077± 3918	27314± 3301	11294± 2175	18517± 1796	27649± 1796	26387 ±2158	16314± 2781
Methanol	31649± 4993	32426± 4627	17180± 4112	21719± 2741	27599± 2657	24748 ±3032	13913± 2433
Ethanol	39686± 3403	34490± 2934	16621± 3057	20135± 2247	26188± 2457	21492 ±3085	14892± 2198
ATV	35863± 4929	33955± 2733	15424± 2406	16170± 2438	23703± 2672	24027 ±2409	11411± 1696
RU486	32124± 3943	35715± 2618	15529± 1923	19714± 1286	29520± 1283	29269 ±1638	19784± 1432
ATV+RU486	39664± 1430	35955± 1426	18540± 2887	24062± 2772	26009± 2822	26032 ±1582	15640± 1075

Table 5.03: Rate Pressure Product. S-stabilization, I-Ischaemia & R-Reperfusion

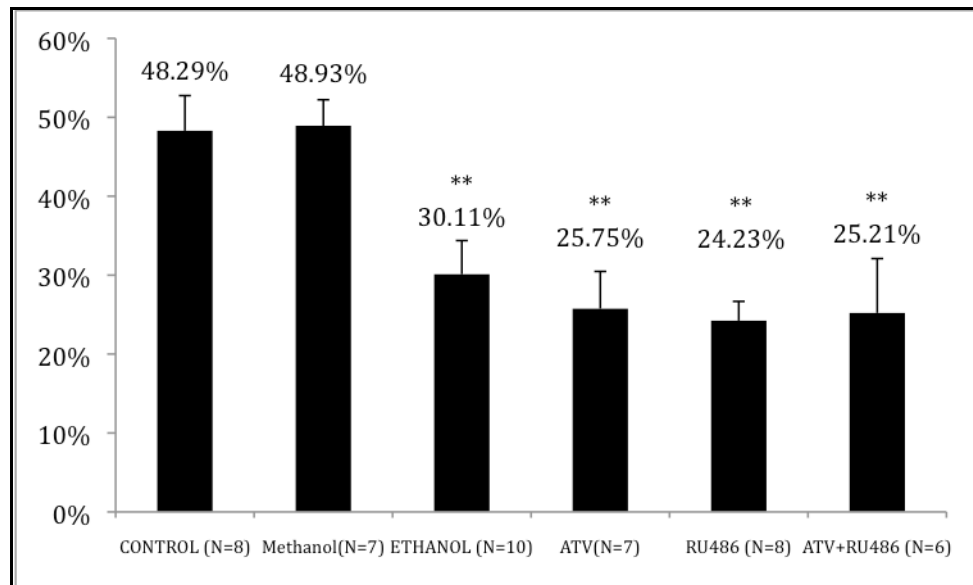


Figure 5.02: When compared with the control, all groups except for the methanol group, showed significant reduction in infarct size including the group perfused with ethanol (vehicle for RU486).

The infarct size in the various groups were as follows: (a) Control 48 ± 5 % (b) Methanol (vehicle for ATV) 49 ± 3 % (c) Ethanol (Vehicle for RU486) 30 ± 4 % (d) Atorvastatin (ATV) 26 ± 5 % (e) RU486 24 ± 2 % (f) ATV + RU486 25 ± 7 %. The ethanol-treated hearts were significantly protected with a p value of <0.05 versus control hearts. The ATV treated hearts and the RU486 treated hearts were significantly protected with p values of <0.05 and <0.01 respectively. Hearts treated with a combination of ATV and RU486 were also significantly protected with a p value <0.05 .

From the above results it would appear that ethanol which was used as the vehicle for RU486 itself is cardioprotective. Thus in order to establish if RU486 abrogated the infarct sparing action of Atorvastatin, the experiments were repeated by dissolving RU486 at the same dose in 0.1% methanol. For this set of experiments, 31 hearts were used. One heart was excluded as it did not attain baseline characteristics at the end of stabilization and infarct data from 4

hearts were excluded due to small infarct size (tables 5.04. 5.05 & 5.06 and Figure 5.03).

Baseline Characteristics

Groups	Number	Weight \pm SEM (gms)
Methanol Control	8	469 \pm 23
Atorvastatin	6	437 \pm 17
RU486	6	479 \pm 38
Atorvastatin + RU486	6	474 \pm 40

Table 5.04: Mean Body weight (gms).

Groups	S10	S30	I5	I30	R5	R30	R120
Methanol- Control	17 \pm 0.9	16 \pm 0.9	7 \pm 0.5	7 \pm 0.5	14 \pm 0.5	12 \pm 0.9	6 \pm 0.6
Atorvastatin	15 \pm 0.6	14 \pm 0.3	7 \pm 0.3	6 \pm 0.5	16 \pm 0.9	12 \pm 2	5 \pm 0.4
RU486	18 \pm 1.5	16 \pm 1	7 \pm 0.6	6 \pm 0.8	17 \pm 2	11 \pm 2	6 \pm 1
Atorvastatin + RU486	18 \pm 1	16 \pm 0.6	8 \pm 0.4	7 \pm 0.6	14 \pm 0.9	10 \pm 1	6 \pm 0.6

Table 5.05: Mean Coronary Flow Rate (ml/min \pm SEM). S-stabilization, I-Ischaemia & R-Reperfusion.

Groups	S10	S30	I5	I30	R5	R30	R120
Methanol- Control	32292 \pm 4266	28237 \pm 2092	12154 \pm 1223	19067 \pm 2245	24434 \pm 2329	23517 \pm 2595	13294 \pm 2091
Atorvastatin	34708 \pm 5670	32439 \pm 3484	15045 \pm 2811	17090 \pm 2672	25630 \pm 2190	25709 \pm 2040	12030 \pm 1868
RU486	35352 \pm 2581	37240 \pm 2384	16603 \pm 3068	20473 \pm 1823	28213 \pm 1725	23999 \pm 2094	15420 \pm 2264
Atorvastatin+RU486	38851 \pm 2657	38461 \pm 2602	20352 \pm 2001	19304 \pm 2987	23947 \pm 2615	23958 \pm 2055	16514 \pm 1636

Table 5.06: Mean Rate Pressure Product. S-stabilization, I-Ischaemia & R-Reperfusion.

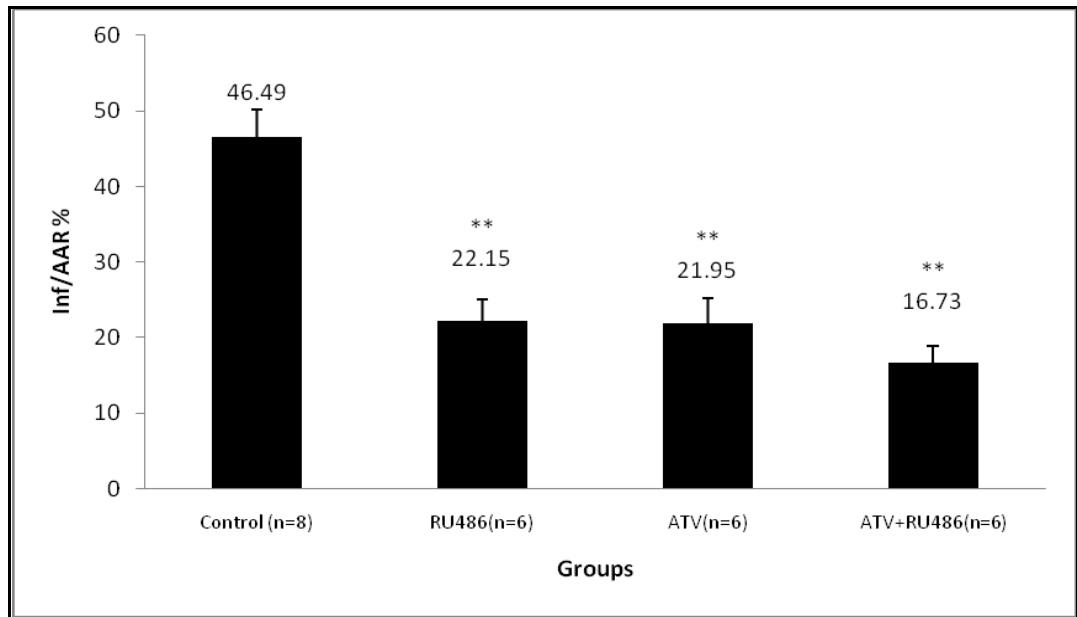


Fig5.03: All groups had significant reduction in infarct size when compared to methanol controls (p value <0.05).

The mean infarct/AAR % for the various groups were as follows:(a) Vehicle (Methanol) control hearts was $47 \pm 4\%$, (b) RU486 treated hearts was $22. \pm 3\%$ (p value <0.001 when compared with control), (c) ATV treated hearts was $22 \pm 3\%$ (p value <0.001 when compared with control) and (d) ATV+RU486 treated hearts was $17 \pm 2\%$ (p value < 0.001 when compared with control).

From these results it is clear that the GCR blocker, RU486, when administered acutely during reperfusion is able to protect the ischaemic-reperfused myocardium. Infact it would seem to be more protective when administered with Atorvastatin although this did not approach significance. As this is the case we were unable to conclude from the infarct size analysis as to whether Atorvastatin acts via the GCR as the pharmacological agent used to block this receptor in itself is cardioprotective.

5.4 DISCUSSION

These results clearly demonstrate that Atorvastatin is cardioprotective when administered at the onset of reperfusion. We were unable to conclude if this action was mediated via the GCR as the GCR antagonist was in itself cardioprotective.

Statins have been suggested to have the same binding affinity towards the glucocorticoid receptor as steroids and Atorvastatin has the same degree of affinity as that of dexamethasone²⁷³. Corticosteroids also acutely protect the heart from reperfusion injury by activating Akt and phosphorylation of GSK-3 β ²⁷⁴. The glucocorticoid, dexamethasone has also been shown to increase the levels of Heat Shock Protein 72 (HSP 72) by inducing its transcription factor, HSF-1 (Heat Shock Factor-1), and thus protect adult rat cardiomyocytes from hypoxia-reoxygenation injury³¹². Atorvastatin has also been shown to phosphorylate HSP27 and thus attenuate reperfusion injury in a mouse ex vivo model²⁶¹. In a mouse Langendorff model, dexamethasone has been shown to be cardioprotective and this was abrogated by the inhibition of COX-2 (Cyclooxygenase-2 thus indicating that the increase in the production of arachidonic acid metabolites was linked to the GCR³¹³. Atorvastatin administered pre-ischaemically to rat hearts was shown to have an infarct sparing action that paralleled the activation of COX-2²⁵⁹. It would appear that statins and steroids are similar in their ability to acutely protect the myocardium and given the affinity of Atorvastatin to the GCR, it would seem reasonable to hypothesize that Atorvastatin-induced cardioprotection was in some way linked to the GCR.

There is conflicting evidence about the downstream effectors of dexamethasone when administered acutely during ischaemia-reperfusion. For example, Langendorff perfused rat hearts were shown to have a smaller infarct size when treated with dexamethasone prior to ischaemia but this protection did not reflect in an increase in phosphorylation of Akt or HSP72 but was possibly related to Erk1/2 activation³¹⁴. Dexamethasone has been shown to increase infarct size in Langendorff perfused rat hearts when administered in the perfusate pre-ischaemically and this effect has been attenuated by the use of the mineralocorticoid receptor antagonist Spironolactone but not the GCR antagonist RU 486³¹⁵. This would suggest that glucocorticoid mediated cardioprotection may not be related to its action on the GCR and also may not be mediated via PI3K/Akt.

There is also evidence to suggest that statins may well antagonize the effects of glucocorticoids and vice versa. Lovastatin has been shown to inhibit the prenylation of the G protein subunits which would thus be able to suppress the transcriptional actions of the GCR³¹⁶. The isoprenoid intermediate, farnesyl pyrophosphate (which is a metabolite of mevalonic acid of the cholesterol biosynthetic pathway), has been shown to act via the GCR and inhibit epithelialisation and thus wound healing. Mevastatin by virtue of inhibiting mevalonate formation, reduces the levels of farnesyl pyrophosphate and thus indirectly inhibits GCR associated activity³¹⁷. Fluvastatin has been shown to induce apoptosis in vascular endothelial cells by inhibiting the prenylation of ras Signal transduction pathway (required for cell survival) and this effect has been reversed by the use of dexamethasone via its interaction with GCR although this inhibition is not specifically targeted at Fluvastatin activity³¹⁸.

Thus despite the simple elegance of our hypothesis, it would seem that the interaction between steroids, statins and GCR activity is quite complex. While there is evidence to suggest that corticosteroid and statins have similar action in protecting the myocardium against the effects of reperfusion injury, there is also evidence that steroids and statins may antagonize each other's effects and also that the mechanism of action of steroid induced cardioprotection may well be via the mineralocorticoid receptor activation as against GCR activation.

These experiments reveal that ethanol used as a solvent for RU486, was protective when administered during reperfusion. There is ample evidence to suggest that acute ethanol administration prior to ischaemia acts via a Protein Kinase C- δ (PKC- δ) which then activates PKC- ϵ which is known to be cardioprotective by inhibition of the MPTP. However this is dependent on the timing of ethanol administration prior to ischaemia, as sufficient time must elapse for the PKC to translocate to the mitochondria³¹⁹. It would thus appear that this is unlikely to be the mechanism of ethanol induced cardioprotection, demonstrated in these experiments. Ethanol has various potential targets such as adenosine receptors, opioid receptors, free radicals etc³²⁰, all of which have been shown to be involved in cardioprotection. It is well known that some of the targets of preconditioning and postconditioning are the same and this may well be one of the reasons that ethanol administered at the onset of reperfusion reduced the infarct size.

In the above mentioned experiments, RU486 was seen to be protective in the setting of myocardial ischaemia-reperfusion. This may be due to the following reasons: (a) The partial agonist activity of RU486. This partial agonist activity of RU486 is dependent on the GCR levels³²¹ and also the N-terminus portion of

the GCR³²². In the presence of high levels of GCR, RU486 acts as an agonist. In the experiments discussed in this thesis, exogenous glucocorticoids were not administered. Thus there might have been significant availability of GCR for the RU486 to bind to, resulting in an agonist activity, (b) The cardioprotection may also be as a result of the inhibition of chronic cortisol associated infarct expansion³²³, (c) The amino acids glutamate and aspartate are released by cardiomyocytes during ischaemia³²⁴ and glutamate has been shown to be cardiotoxic in H9C2 myocytes. RU486 pre-treatment is said to reduce this cardiotoxic effect and may well be a potential mechanism of RU486 induced cardioprotection³²⁵, and (d) RU486 has also been shown to possess antioxidant properties that may well mop up the harmful ROS³²⁶ (Reactive Oxygen Species).

5.5 CONCLUSION

The data presented demonstrates that Atorvastatin when administered acutely during reperfusion reduces infarct size expansion due to reperfusion injury in isolated Langendorff perfused rat hearts. However the GCR antagonist, RU486, was in itself cardioprotective and thus it was not possible to conclude as to whether Atorvastatin induced cardioprotection was associated with GCR activation. These experiments could not be repeated with an alternative GCR blocker as the only other antagonist is currently unavailable. However these experiments could be potentially performed in a different model such as a mouse 'knock-out' model in which the GCR responsive elements are inactive³²⁷.

6. ESTABLISHING THE ROLE OF ADENOSINE IN ATORVASTATIN MEDIATED PROTECTION AGAINST REPERFUSION INJURY

6.1 AIMS AND HYPOTHESIS

Adenosine is a well known cardioprotective agent and has been implicated in ischaemic preconditioning³²⁸⁻³³¹. There is also evidence to suggest that statin mediated cardioprotection involves adenosine, when statins have been administered prior to ischaemia^{246-230,332-334}. We hypothesized that Atorvastatin administered during reperfusion, mediated its cardioprotection by involving adenosine and that this effect could be blocked by the adenosine receptor antagonist, 8-sulpho phenyl theophylline (SPT). We tested this using a Langendorff rat heart model to assess infarct size following ischaemia and reperfusion in the presence of atorvastatin administered prior to ischaemia and at reperfusion.

6.2 METHODS

Langendorff perfused rat hearts were used and the various groups were subjected to 35 minutes of ischaemia and 120 minutes of reperfusion as discussed in chapter 3. 51 hearts were used of which 5 were excluded as they did not attain acceptable baseline parameters during stabilization, 5 hearts were excluded due to precipitation of Atorvastatin in the rig and a further 10 were excluded due to small AAR or small infarct size, which did not meet the predefined inclusion criteria. Results were expressed as Infarct size/AAR \pm SEM % and the results were tested for significance with one way Anova test followed by Tukey's test (p value <0.05).

The hearts were divided into the following 4 groups. In each case the drugs or vehicle were administered for the last 5 minutes of ischaemia and the first 15 minutes of reperfusion only (see **Figure 6.01**):

(a) *Controls*: Perfused with methanol (0.1%).

(b) *Atorvastatin*: Hearts perfused with atorvastatin at a dose of 50 $\mu\text{mol/l}$.

(c) *SPT*: Hearts were perfused with the non specific adenosine receptor antagonist, 8- Sulfophenyl theophylline (SPT), during reperfusion. This was used at a dose of 100 $\mu\text{mol/L}$ and it was dissolved in the buffer directly. SPT has been used at various doses ranging from 10 $\mu\text{mol/L}$ to 100 $\mu\text{mol/L}$. However a dose of 100 $\mu\text{mol/l}$ has been shown to antagonize the effect of adenosine receptors in isolated perfused rat hearts^{335,336}.

(d) A combination of *ATV and SPT* at a dose of 50 $\mu\text{mol/l}$ and 100 $\mu\text{mol/l}$ respectively. SPT was perfused for 2 minutes before the combination of the ATV and SPT. This was so as to allow the SPT to reach and inhibit the adenosine receptors prior to the introduction of ATV.

Protocol

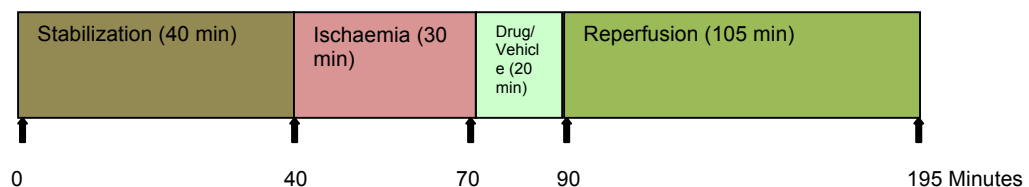


Fig6.01: Protocol: Drug: ATV, SPT, ATV+SPT. Vehicle: Methanol (Control)

6.3 RESULTS

6.3.1 ATORVASTATIN AND SPT AT REPERFUSION

The baseline parameters for all hearts were similar except in the case of the hearts perfused with the combination of ATV and SPT, in which the developed pressure and RPP were significantly higher when compared to the methanol (control) group. Again as all drug therapy was at the onset of reperfusion, this difference is not related to drug therapy (**tables 6.01,6.02 and 6.03 and figure 6.02**).

Baseline Characteristics

Groups	Number	Body Weight (gms \pm SEM)
Methanol (Control)	7	464 \pm 13
Atorvastatin (ATV)	9	433 \pm 17
SPT	7	410 \pm 27
ATV+SPT	8	408 \pm 27

Table 6.01: Body weight (gms \pm SEM).

Groups	S10	S30	I5	I30	R5	R30	R120
Methanol(Control)	16 \pm 1	15 \pm 1	8 \pm 0.4	7 \pm 0.5	14 \pm 0.5	12 \pm 1	6 \pm 0.7
ATV	16 \pm 1	15 \pm 0.5	7 \pm 0.2	7 \pm 0.4	15 \pm 0.6	12 \pm 1	6 \pm 0.7
SPT	17 \pm 1	16 \pm 1	6 \pm 0.3	6 \pm 0.3	14 \pm 0.6	12 \pm 1	7 \pm 1
ATV+SPT	16 \pm 1	15 \pm 1	6 \pm 0.5	6 \pm 0.4	12 \pm 1	11 \pm 1	6 \pm 0.6

Table 6.02: Coronary flow rate (ml/min). S-stabilization, I-Ischaemia & R-Reperfusion.

Groups	S10	S30	I5	I30	R5	R30	R120
Methanol (Control)	31234±4773	27795±2311	13356±1757	19649±2021	26797±2255	24667±2872	14704±2890
ATV	36237±3757	34335±2128	16594±2090	20693±3188	27403±1915	26871±1759	15295±2383
SPT	37791±3879	35502±3227	16162±2196	18326±1927	28293±1248	27446±2216	19471±1558
ATV+SPT	46191±3648*	46191±3648*	17185±2628	23449±1515	25977±1170	28503±2810	20464±1967

Table 6.03: Rate pressure product. S-stabilization, I-Ischaemia & R-Reperfusion.

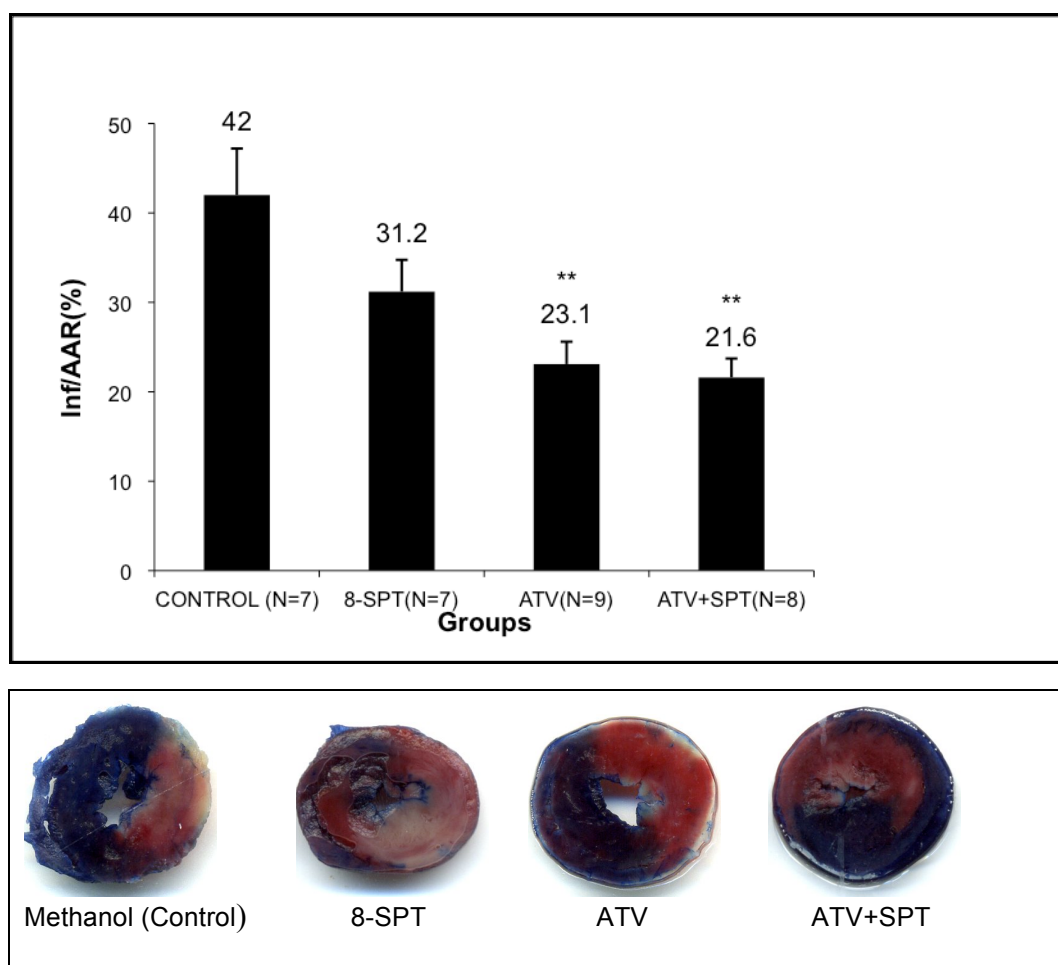


Figure 6.02: Infarct/AAR for ATV+SPT administered at reperfusion. **p<0.05 when compared to control.

The Infarct/AAR in the four groups was as follows: control- 42±5%, SPT-31±4, ATV- 23±3 (P <0.01 when compared with control) & ATV+SPT- 22±2 (p value <0.01 when compared with control). The ATV and ATV+SPT group had significantly lower infarct sizes when compared to controls (p value <0.01).

It is thus clear that the protection obtained by acute administration of Atorvastatin during reperfusion, is not abrogated by the non-selective adenosine receptor antagonist 8-SPT. In order to explore the possibility of different mechanisms of Atorvastatin-induced acute cardiovascular protection in two different settings i.e, prior to ischaemia and at reperfusion, experiments were performed to measure infarct size in Langendorff perfused rat hearts when Atorvastatin was administered prior to ischaemia.

6.3.2 ATORVASTATIN ADMINISTERED PRIOR TO ISCHAEMIA

The hearts were again divided into four groups as follows:

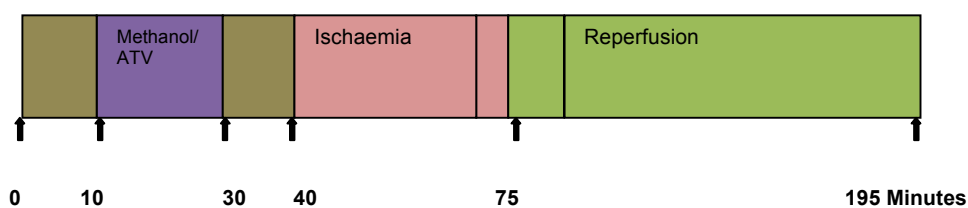
(a) *Controls (Methanol)*: Perfused with methanol (0.1%). Following the first 10 minutes of stabilization, methanol dissolved in buffer was perfused for 20 minutes followed by a 10 minute wash out period, prior to ischaemia (**Figure 6.03: Protocol A**).

(b) *Atorvastatin*: Perfused with Atorvastatin at a dose of 50 $\mu\text{mol/L}$. The protocol was similar to that for the control hearts (**Figure 6.03: Protocol A**).

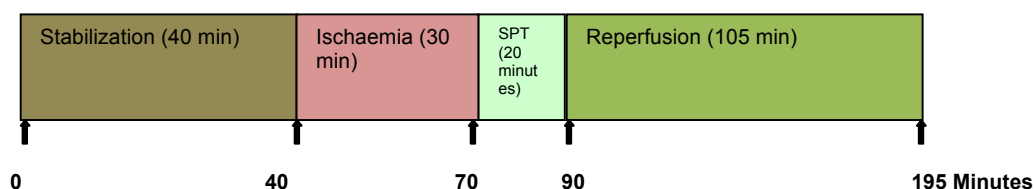
(c) *SPT*: SPT dissolved in buffer was perfused at a dose of 100 $\mu\text{mol/L}$ during the last 5 minutes of ischaemia and the first 15 minutes of reperfusion (**Figure 6.03: Protocol B**).

(d) *Atorvastatin + SPT*: In this protocol, Atorvastatin was perfused prior to ischaemia as mentioned above and SPT was perfused during reperfusion as mentioned above (**Figure 6.03: Protocol C**).

A.



B.



C.

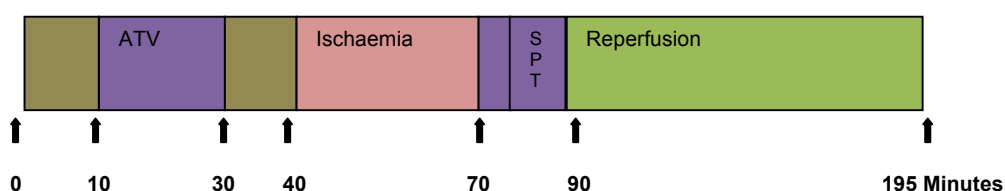


Figure 6.03: Protocols for Atorvastatin administration prior to ischaemia.

28 hearts were used of which 2 were excluded due to poor heart function during stabilization and 2 hearts were not analysed as there was no washout period between drug administration and ischaemia. There was no significant difference between the groups in baseline characteristics. (tables 6.04, 6.05 and 6.06).

Baseline Characteristics

Groups	Number	Body Weight (gms \pm SEM)
Methanol (Control)	5	364 \pm 24
Atorvastatin (ATV)	5	364 \pm 22
SPT	6	365 \pm 23
ATV+SPT	6	408 \pm 9

Table 6.04: Mean Body Weight (gms \pm SEM).

Groups	S10	S30	I5	I30	R5	R30	R120
Methanol(Control)	19 \pm 0.7	17 \pm 0.8	7 \pm 0.5	7 \pm 0.5	14 \pm 0.5	13 \pm 0.5	8 \pm 0.9
ATV	18 \pm 1	18 \pm 0.5	7 \pm 0.9	7 \pm 0.9	14 \pm 0.9	13 \pm 0.4	8 \pm 0.8
SPT	17 \pm 0.7	16 \pm 0.8	8 \pm 0.5	8 \pm 0.6	13 \pm 0.3	12 \pm 0.7	8 \pm 0.9
ATV+SPT	20 \pm 0.7	19 \pm 1	7 \pm 0.8	7 \pm 0.9	14 \pm 1	12 \pm 1	8 \pm 0.8

Table 6.05: Mean Coronary flow rate (ml/min \pm SEM). S-stabilization, I-Ischaemia & R-Reperfusion.

Groups	S10	S30	I5	I30	R5	R30	R120
Methanol (Control)	50136 \pm 2657	45106 \pm 1846	24976 \pm 1757	27328 \pm 2390	32306 \pm 1191	34670 \pm 1731	26935 \pm 909
ATV	45948 \pm 3456	47154 \pm 2857	19006 \pm 2090	26697 \pm 2872	32682 \pm 2333	33965 \pm 1864	28304 \pm 1757
SPT	45059 \pm 5947	43016 \pm 4943	23250 \pm 2196	29722 \pm 4505	33308 \pm 2624	33235 \pm 2070	23762 \pm 2414
ATV+SPT	48012 \pm 4270	41105 \pm 5998	20405 \pm 2628	26473 \pm 4978	34098 \pm 4828	33702 \pm 5502	26031 \pm 3903

Table 6.06: Mean Rate Pressure Product. S-stabilization, I-Ischaemia & R-Reperfusion.

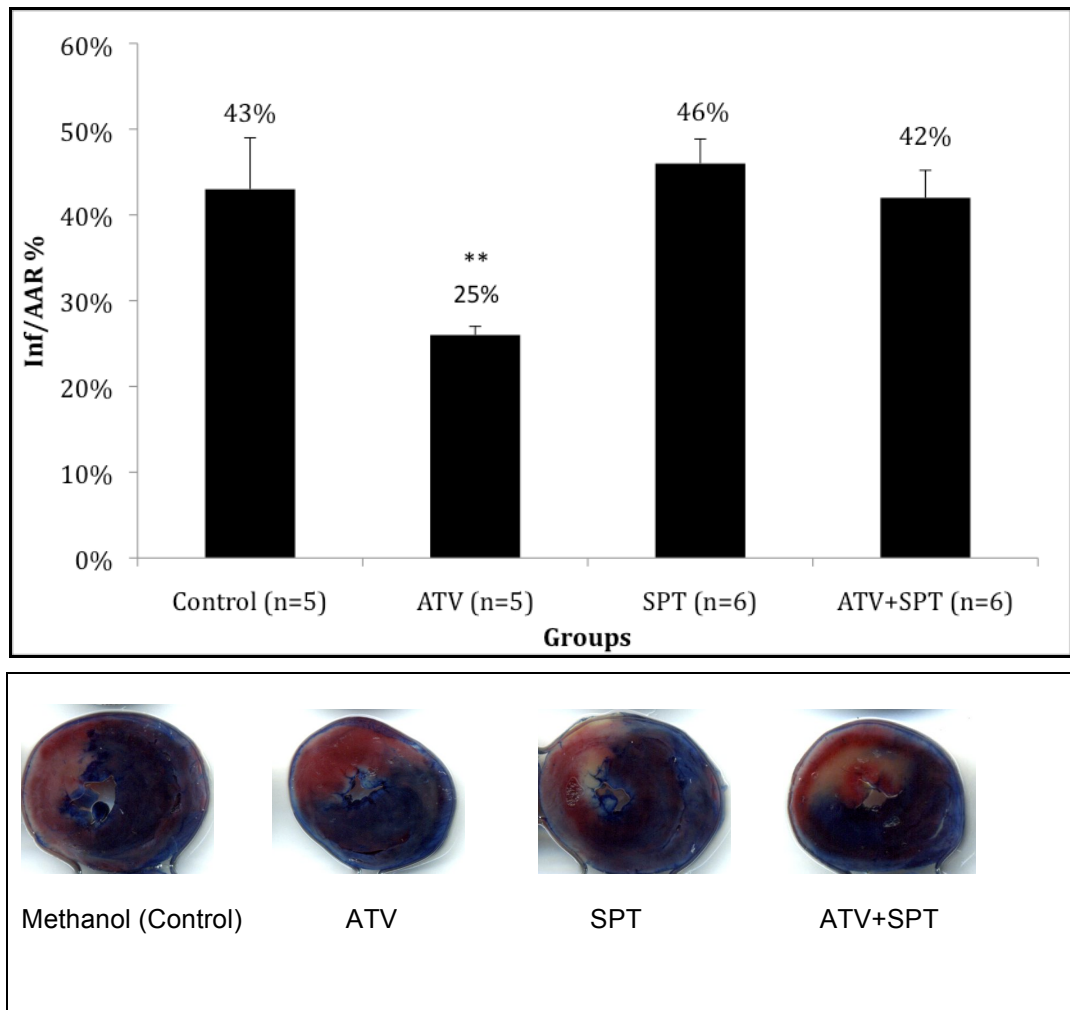


Figure 6.04: Infarct/AAR % with pre-ischaemic administration of ATV. ** p value <0.05 when compared with control.

The mean infarct sizes as a percentage of the AAR \pm SEM for the various groups were as follows: (a) Control: $43 \pm 6\%$ (b) SPT: $46 \pm 3\%$ (c) ATV: $25 \pm 1\%$ (p value <0.05 when compared with control) (d) ATV+SPT: $42 \pm 3\%$ (p value <0.05 when compared with ATV). It is thus clear that Atorvastatin is cardioprotective even when administered acutely prior to ischaemia and this protection is blocked by the adenosine receptor antagonist, SPT.

6.4 DISCUSSION

These experiments thus demonstrate that Atorvastatin is cardioprotective when administered prior to ischaemia and at the onset of reperfusion. However SPT is able to abrogate the protection of pre-ischaemic administration of Atorvastatin

thus demonstrating the role of adenosine at reperfusion but is unable to abrogate the protection associated with administration of Atorvastatin at the onset of reperfusion.

Adenosine is a purine nucleoside which is widely distributed in tissues and plays a central role in the energy metabolism of the cell³³⁷. Its formation is closely related to the energy consumption of the cell³³⁷. High levels of adenosine accumulate in the extra cellular space during ischaemia/hypoxia and its levels gradually subside during reperfusion³³⁸. It is produced by the action of 5' nucleotidase which dephosphorylates Adenosine Mono Phosphate (AMP) and the hydrolysis of S-Adenosyl homocysteine³³⁹ (**Figure 6.05**).

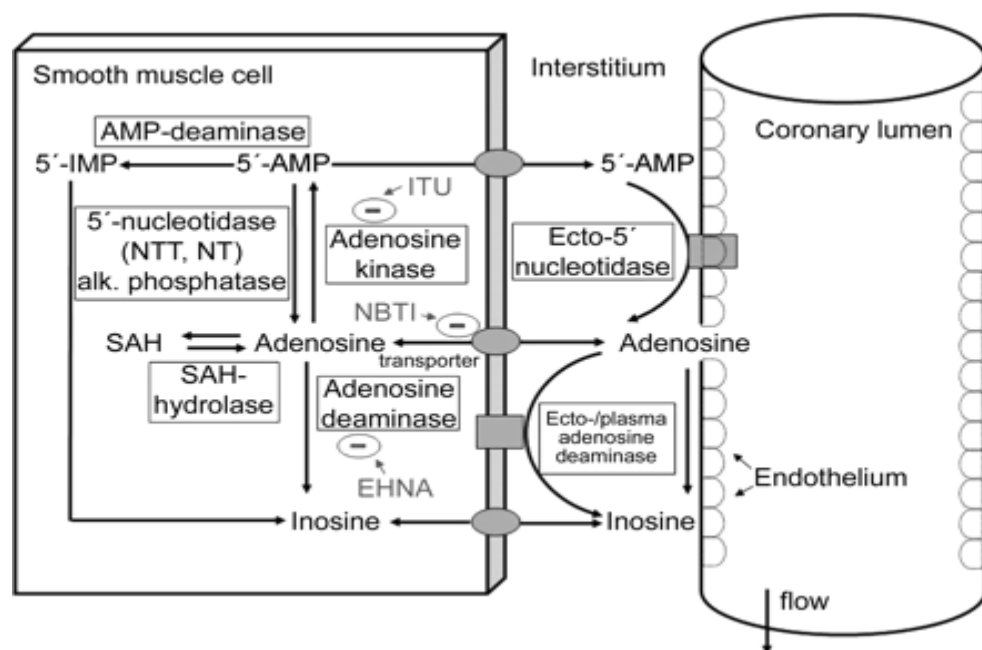


Figure 6.05: Adenosine is produced by the action of ecto 5'nucleotidase on adenosine mono phosphate (AMP) and the action of S-adenosyl homocysteine hydrolase (SAH-hydrolase) on SAH. Adenosine is degraded to inosine by adenosine deaminase which is subsequently converted to hypoxanthine. IMP-Inosine Mono Phosphate, ITU: iodotubercidine, blocks adenosine kinase and thus inhibits the conversion of adenosine to AMP, NBTI: n-nitrobenzyl thioinosine, blocks adenosine membrane transport, EHNA: erythro-6 (2- hydroxy-3-nonyl)-adenine, blocks adenosine deaminase and thus inhibits the degradation of adenosine. Taken from Frobert et al³⁴⁰.

There are numerous studies to show that adenosine is cardioprotective and these have been discussed in detail in two excellent reviews^{178,341}. I will discuss a few relevant examples as follows. Adenosine has been shown to reduce infarct size when administered to isolated perfused rabbit hearts, prior to ischaemia and this mirrored the protection obtained by the preconditioning protocol³³¹. In the same group of experiments, the non-specific adenosine receptor antagonist was shown to abrogate the infarct size limitation of preconditioning³³¹. Further confirming the role of adenosine, in isolated perfused rat hearts, a preconditioning cycle increased the amount of adenosine measured by microdialysis³²⁹. It was subsequently demonstrated that Ischaemic Preconditioning (IPC) was mediated through A_{2B} adenosine receptors and was dependent on an increase in ecto5'nucleotidase activity (responsible for production of adenosine), in a transgenic A_{2B} receptor deficient mouse model³²⁸. In Langendorff perfused rabbit hearts it was demonstrated that ischaemic preconditioning (and thus adenosine) activated Protein Kinase C (PKC) which interacted with adenosine A_{2B} receptors during reperfusion and stimulated Akt/Erk1/2 components of the RISK pathway³³⁰.

As already discussed in section 1.4.1, statins administered preischaeemically in a dog invivo model reduced infarct size and this was mediated by activation of ecto5'nucleotidase after the first 15 minutes of reperfusion, which in turn was associated with Akt activation²⁴⁷. As mentioned previously, recently it has been demonstrated by Western blots in rat hearts that statin activation of ERK1/2 is dependent on PI3 Kinase induced increase in ecto5'nucleotidase which then increases extracellular levels of adenosine resulting in interaction with the adenosine receptors (including A_{2b})²⁴⁸. Pravastatin administered to rabbit hearts prior to ischaemia has been shown to have a PKC dependent, infarct

sparing action³³². In human endothelial cells Atorvastatin was shown to accelerate the degradation of nucleotides and increase the production of adenosine and this was shown to be associated with an increase in the activity of ecto5'nucleotidase³³⁴. As already discussed Pravastatin has been shown to have some interaction with adenosine metabolism when administered to patients prior to angioplasty²⁴⁶. In a double blind study with 21 human volunteers, Rosuvastatin administered prior to induction of forearm ischaemia demonstrated an improved vasodilator effect to dipyridamole (decreases endocytotic turnover of adenosine and thus increases extracellular adenosine) and this effect was abolished by the adenosine antagonist caffeine³³³. This study showed some indirect evidence that the augmentation in the level of endogenous adenosine was probably due to the effect of Rosuvastatin on ecto5'nucleotidase.

Adenosine production in the interstitial compartment increases under conditions of stress such as ischaemia and these levels gradually normalize with reflow³³⁸. The extracellular adenosine is recycled to form AMP by adenosine kinase, when a sufficient concentration gradient has been established and under well oxygenated condition³⁴². However the low pH and hypoxia associated with ischaemia results in inhibition of adenosine kinase and an increase in the extracellular levels of adenosine. The extracellular production of adenosine under conditions of hypoxia is linked to the activity of ecto5'nucleotidase. Indeed in experiments with vascular endothelial cells, hypoxia has been shown to increase the cell surface expression of ecto5'nucleotidase over time and also increase its availability on the cell surface by reducing the endocytotic turnover³⁴³. This increase in the activity of ecto5'nucleotidase and thus availability of adenosine does not necessarily translate into cardioprotection and

an additional stimulus such as preconditioning³³¹, adenosine infusion (pre-ischaemia)³²⁹ or postconditioning³⁴⁴ is required to enhance the affinity of adenosine to its receptor targets.

Adenosine acts via the A₁, A_{2A}, A_{2B} and A₃ receptor subtypes. The binding of adenosine to A₁ and A₃ receptors reduces c-AMP levels while its binding to A_{2A} & A_{2B} receptors enhances adenylyl cyclase activity and thus increase the production of intracellular c-AMP³⁴⁰. A₁ and A₃ receptors increase the levels of PKC³⁴⁰ (**figure 6.06**). Adenosine induced cardioprotection due to preconditioning was shown to be mediated by A₁ receptors in in vivo rabbit hearts³⁴⁵. In a transgenic mouse knock out model it was demonstrated that the preconditioning effect was mediated via ecto5'nucleotidase and A_{2B} receptors³²⁸.

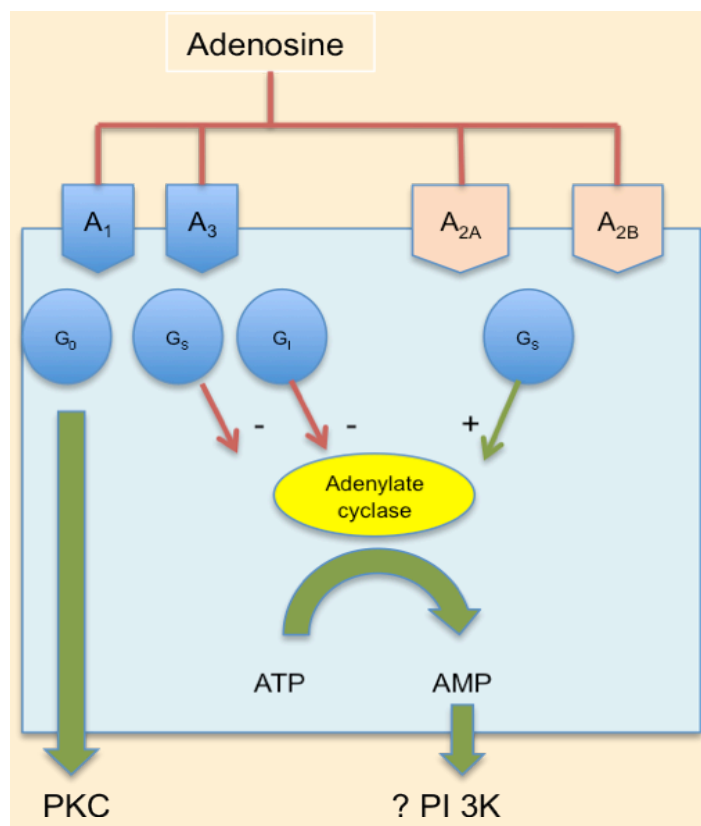


Figure 6.06: Adenosine receptors and effectors. +: Activates Adenylate cyclase and -: Inhibits Adenylate cyclase. Adapted from Aderis pharmaceuticals.

The role of adenosine in the reperfusion phase is unclear with conflicting evidence. Intravenous adenosine and receptor agonists have been shown to be protective when given acutely during reperfusion in a rabbit *in vivo* model and a dog *in vivo* model^{346,347}. On the other hand in the same species with similar *in vivo* models, adenosine administered at reperfusion has not been shown to be protective^{348,349}. The AART trial in rabbits with A1 receptor agonist administered *in vivo* at reperfusion did not demonstrate any cardioprotection³⁵⁰. The clinical trials AMISTAD 1 & 2 did demonstrate some reduction in infarct size in specific patient sub-groups but did not offer any prognostic benefit^{180,181}. AMISTAD was a prospective, open-label trial of thrombolysis with randomization of 236 patients to adenosine or placebo within 6 hours post infarction. The primary end point was infarct size reduction measured by Tc-99 sestamibi SPECT scan 5-7 days post infarction and secondary end point was myocardial salvage index and composite of in-hospital outcomes. Patients with an anterior infarct who received adenosine had a 67% relative reduction in infarct size and were more likely to reach the composite clinical outcomes. AMISTAD-2 trial was a randomized multicentre double-blinded placebo-controlled trial of adenosine as an adjunct for reperfusion in the treatment of acute myocardial infarction. 2118 patients were recruited and infarct size was measured in 236 patients with Tc-99 sestamibi SPECT. The pooled adenosine group had a smaller infarct size and this positively correlated with fewer clinical outcomes.

Given the evidence it would appear that adenosine induced cardioprotection is dependent on pre-ischaemic augmentation of adenosine levels which in turn increases the activity of PKC and also increases the levels of ectonucleotidase, resulting in A2B mediated activation of the RISK pathway at reperfusion.

A2B receptors however have low affinity for adenosine. PKC activation in the first few minutes of reperfusion has been shown to be essential for cardioprotection by preconditioning (and thus adenosine mediated cardioprotection)³⁵¹. Preconditioning would increase the levels of PKC which would then increase the affinity of the A2B receptor for endogenous adenosine and thus trigger cardioprotection through the RISK pathway³³⁹. Initial increase in PKC activity is dependent on adenosine A1 and A3 receptors which couple directly with PKC via phospholipase activation³⁵². Thus the implication is that any agent (in this case Atorvastatin) that were to mimic preconditioning induced enhanced sensitivity of adenosine to the A2B receptor, will have to be administered sufficiently early for the PKC activation to take place (**figure 6.07**).

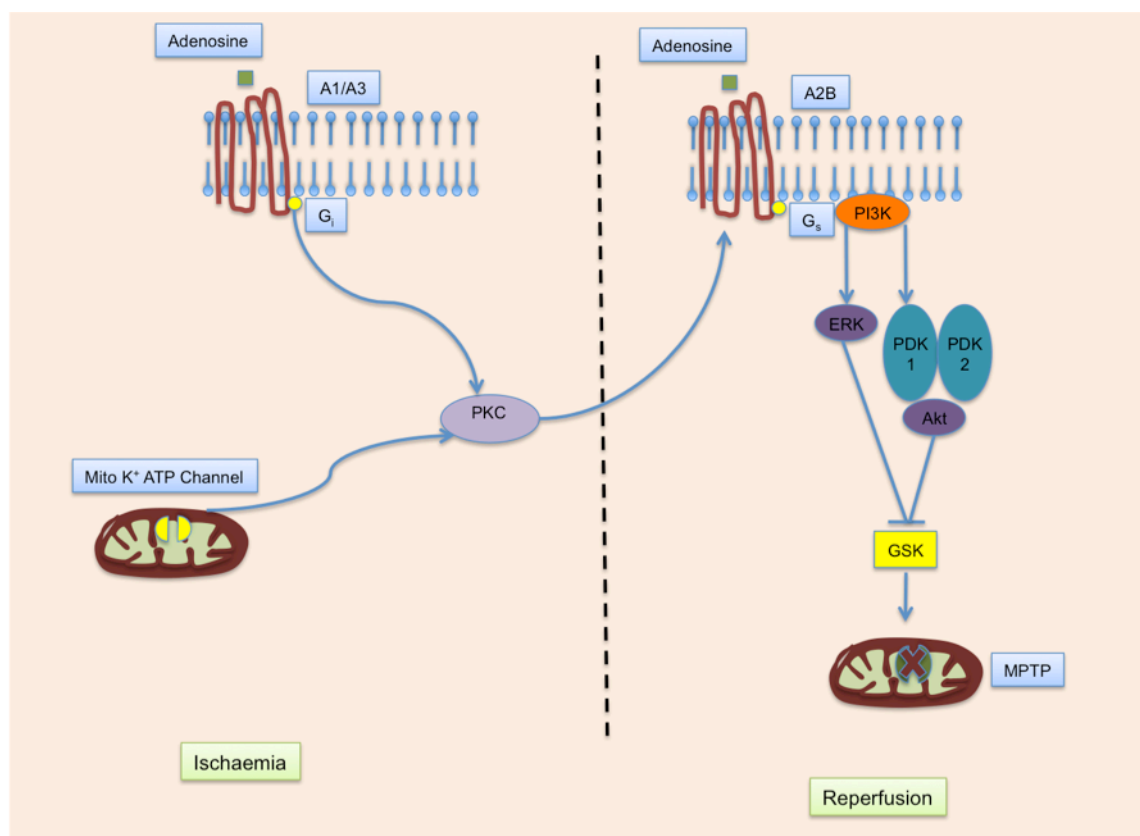


Figure 6.07: Adenosine and cardioprotection. Adenosine acts via the A1 and A3 receptors to increase the levels of Protein Kinase C (PKC) during ischaemia and this in turn enhances the sensitivity of A2B receptors to adenosine which subsequently activates the RISK pathway. Adapted from Cohen et al³³⁹.

As previously discussed statins enhance the levels of ecto5'nucleotidase, adenosine levels and PKC activity when administered prior to ischaemia. This effect may well mimic the preconditioning induced PKC dependent cardioprotection. This would also imply that statins need to be administered sufficiently prior to ischaemia to activate PKC and enhance the affinity of the A2B receptors to adenosine which in turn activate the RISK pathway. However as discussed in previous sections, the experiments in dog in vivo model do show that ecto5'nucleotidase levels rise only after 15 minutes of reperfusion and that this is important for the cardioprotection even of statins, administered prior to ischaemia in these experiments²⁴⁷. This is the only experimental evidence to suggest that in addition to its activity during ischaemia, ectonucleotidase activity is maintained in reperfusion. In the same group of experiments, the activation of ecto5'nucleotidase was dependent on PI3 Kinase activation which occurs during the first few minutes of reperfusion. It could thus be argued that pre-ischaemic delivery of statins increase the activity of PKC during ischaemia and this primes the A2b receptors to the effects of adenosine which in turn is responsible for the activation of PI3 kinase and ecto5'nucleotidase during reperfusion. This further increases the levels of adenosine which in turn activates the RISK pathway.

In the current study, SPT was shown to abrogate the infarct size reduction of pre-ischaemic delivery of Atorvastatin while it did not have any effect on the infarct size reduction of Atorvastatin administered at reperfusion. This may be due to the following reasons:

(a) Pre-ischaemic Atorvastatin possibly activates ectonucleotidase and PKC which in turn enhances the affinity of the A2B receptors for endogenous adenosine but Atorvastatin administered during reperfusion has skipped the

PKC activation mediator phase (and thus the A2B activation) and is thus unable to directly interact with the adenosine receptor. This is backed by previous work which has demonstrated that adenosine or its agonists are ineffective when administered during reperfusion^{345,348-350,353-355}. This also brings to fore the fact that there are probably multiple mechanisms by which statins are cardioprotective.

(b) It may be due to species difference. Ecto5'nucleotidase is abundant in rat cardiomyocytes but not so in the arterial myocytes³⁵⁶. The concentration of 5'-nucleotidase in the pericytes of endothelium is higher in dogs and guinea pigs when compared to rats³⁵⁷. However dog myocardium was not reactive to 5'nucleotidase. This difference in the relative concentration of the enzyme between different compartments of the same tissue type and also between different species may contribute to differences in adenosine metabolism.

(c) In addition, in my studies, Atorvastatin was perfused for a period of only 20 minutes , 15 minutes of which were in the reperfusion phase. This maybe the reason, that we did not observe the benefit of delayed ectonucleotidase activation that has been reported in dogs wherein the enzyme is activated only after 15 minutes of reperfusion²⁴⁷.

6.5 CONCLUSION

These results thus demonstrate that Atorvastatin is able to reduce infarct size when administered prior to ischaemia and during reperfusion. The protection seen with pre-ischaemic atorvastatin is lost when co-administered with SPT, suggesting involvement of adenosine metabolism. However this cardioprotection is not blocked by SPT when Atorvastatin is administered during reperfusion suggesting a non-adenosine dependent unrelated mechanism may be involved.

7. ATORVASTATIN, MEVALONIC ACID AND RHO A METABOLISM

7.1 AIMS AND HYPOTHESIS

Statins inhibit HMG CoA reductase, the rate limiting enzyme in the cholesterol biosynthetic pathway²¹³. As already mentioned statins reduce infarct size when administered during reperfusion by activating the RISK pathway⁸. Statins also inhibit the prenylation of Rho A and thus inhibit the activation of Rho Kinase (ROCK)¹⁴. Rho A and ROCK are inhibitors of PI3K and eNOS¹⁴. We thus hypothesised that Atorvastatin administered acutely during reperfusion protects the heart by inhibiting Rho A. In order to demonstrate this, mevalonic acid was used. Mevalonic acid is a precursor of cholesterol and isoprenoid synthesis and is the rate limiting step in the synthesis of isoprenoids. We used infarct size measurements and Western blotting for Rho A activation.

7.2 METHODS

Langendorff perfused Sprague –Dawley rat hearts were subjected to one of the following protocols: One set of experiments for infarct size analysis was carried out as per standard ischaemia–reperfusion protocol with 40 minutes of stabilization, 35 minutes of ischaemia and 120 minutes of reperfusion. In this

set of experiments vehicle and drugs were administered during the last five minutes of ischaemia and for the first 15 minutes of reperfusion. In the second set of experiments the Langendorff perfused hearts were subjected to 40 minutes of stabilization, 35 minutes of ischaemia and 15 minutes of reperfusion with vehicle and drug perfused for the last 5 minutes of ischaemia and 15 minutes of reperfusion. At the end of 15 minutes of reperfusion, the suture around the LAD was tied and a small quantity of Evan's blue was injected to just about delineate the area at risk and the risk area was quickly excised and snap frozen in liquid nitrogen. The samples were then processed as per Western blot technique, which has been discussed in section 3.

For infarct size analysis the following groups were included:

(a) *Control (Vehicle)*: Methanol in Krebs buffer at a dose of 0.1 % was perfused during reperfusion as per protocol A in **figure 7.01**.

(b) *Atorvastatin*: Atorvastatin dissolved in methanol and buffer was perfused at a dose of 50 $\mu\text{mol/l}$ as per protocol A in **figure 7.01**.

(c) *Mevalonic acid*: Mevalonolactone was purchased from Sigma–Aldrich chemicals. Mevalonolactone was converted to mevalonic acid by dissolving it in 0.1 M sodium hydroxide and then heating it at 50 °c for 2 hours. To this was added 0.1 M hydrochloric acid and the pH was adjusted to 7.4³⁵⁸. Mevalonic acid was used at a dose of 1 mmol/l. This dose has been shown to inhibit the effect of statins on NO activity in porcine arterioles³⁵⁹. Mevalonic acid dissolved in buffer was perfused as per protocol A in **figure 7.01**.

(d) *Atorvastatin + Mevalonic Acid*: The combination of drugs were dissolved in buffer and perfused as per protocol A in **figure 7.01**.

Infarct size analysis was carried out as described before. Results were expressed as Mean Infarct/AAR % \pm SEM and tests of significance were carried out with one way ANOVA testing and tukey post test comparison using Graphpad Prism software (version 5.0) with a p value of <0.05 considered significant.

The following groups were used for Western blot analysis:

(a) *Control (Vehicle)*: Methanol in Krebs buffer at a dose of 0.1 % was perfused during reperfusion as per protocol B in **figure 7.01**.

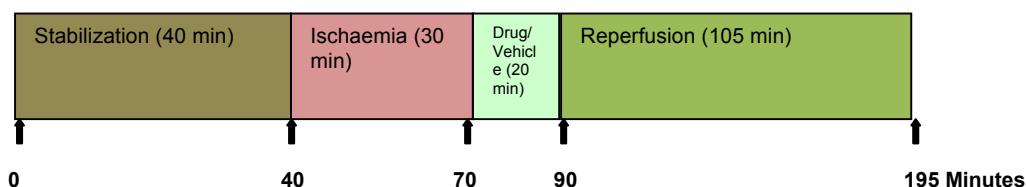
(b) *Atorvastatin*: Atorvastatin dissolved in methanol and buffer was perfused at a dose of 50 μ mol/l as per protocol B in **figure 7.01**.

(c) *Mevalonic acid*: Mevalonic acid was dissolved in buffer at a dose of 1 mmol/l and perfused as per protocol B in **figure 7.01**.

(d) *Atorvastatin + Mevalonic Acid*: The combination of drugs were dissolved in buffer and perfused as per protocol B in **figure 7.01**.

The samples were then processed as per the protocol for Western blot analysis and the relative density of the bands was measures and test of significance was carried out using one way ANOVA with a p value of <0.05 considered significant.

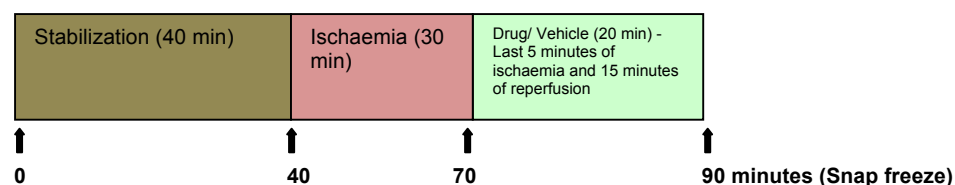
Protocol A:



Drug: Atorvastatin, Mevalonic acid, ATV+ Mevalonic acid

Vehicle: Methanol

Protocol B



Drug: Atorvastatin, Mevalonic acid, ATV+ Mevalonic acid

Vehicle: Methanol

Figure 7.01: Experiment protocols.

7.3 RESULTS

7.3.1 REPERFUSION SALVAGE- IINFARCT SIZE

36 Sprague –Dawley rats were used for this set of experiments. 5 hearts were excluded due to technical reasons and 5 were excluded due to criteria explained in previous sections.

Baseline Characteristics:

Group	Number	Body Weight.
Control	8	432±23
ATV	6	421±14
Mevalonic Acid	6	447±29
ATV+ Mevalonic acid	6	438±13

Table 7.01: Mean Body Weight (gms± SEM)

Group	S10	S30	I5	I30	R5	R30	R120
Methanol (Control)	16 ± 0.9	15 ± 0.7	7 ± 0.3	7 ± 0.4	14 ± 0.4	12 ± 1	7 ± 0.7
ATV	17 ± 1	15 ± 0.5	6 ± 0.4	6 ± 0.4	16 ± 1	11 ± 0.9	6 ± 1
Mevalonic Acid	18 ± 0.6	17 ± 1	8 ± 0.6	8 ± 0.6	15 ± 1	12 ± 0.9	7 ± 0.9
ATV+Mevalonic Acid	17 ± 1	15 ± 1	6 ± 0.8	6 ± 0.6	17 ± 1	12 ± 1	8 ± 0.9

Table 7.02: Mean Coronary Flow Rate (ml/min ± SEM). S-Stabilization, I –Ischaemia, R-Reperfusion.

Groups	S10	S30	I5	I30	R5	R30	R120
Methanol (Control)	33501± 4592	30334± 3237	14395± 1837	20710± 1927	26818± 2214	25548± 2693	16249± 3134
ATV	40328± 2787	35526± 1698	16281± 1917	20801± 2170	26305± 2101	25886± 1580	17629± 932
Mevalonic acid(MEV)	38881± 3745	35290± 3039	19653± 2910	23949± 2430	26616± 2454	26784± 2520	20193± 3298
ATV+MEV	42873± 4536	38718± 4186	16235± 3314	23656± 3601	23991± 3497	29295± 3750	23009± 2613

Table 7.03: Mean Rate Pressure Product. S-Stabilization, I –Ischaemia, R-Reperfusion.

Infarct Size

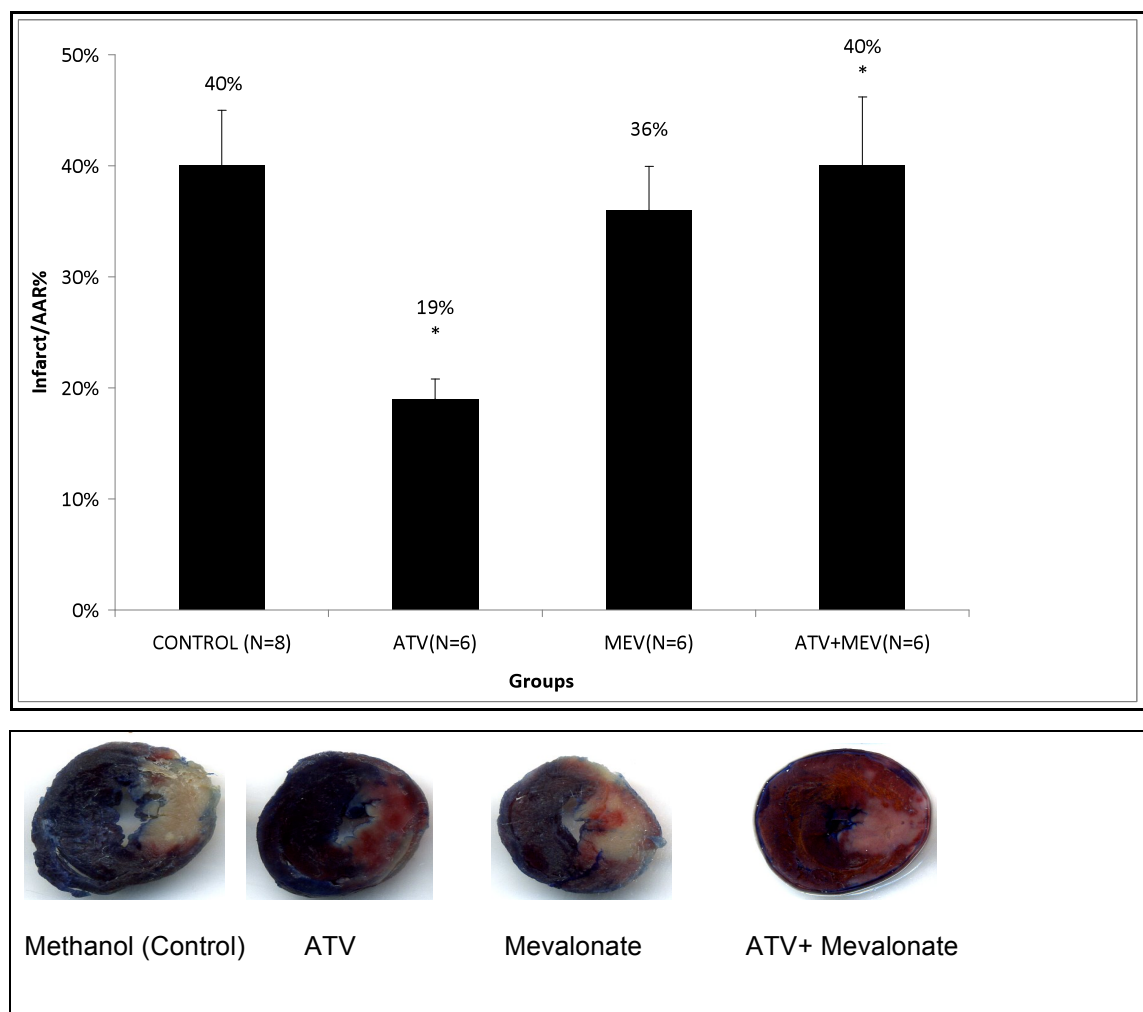


Fig 7.02: Hearts treated with Atorvastatin were significantly protected when compared to vehicle control (p value <0.05) and this protection was abolished by the co-administration of mevalonic acid (p value < 0.05 when compared with ATV) .

The mean infarct size \pm SEM for the groups were as follows: Control $40 \pm 5\%$, Atorvastatin $19 \pm 2\%$ (p <0.05 when compared with control), Mevalonic acid $36 \pm 4\%$ and Atorvastatin + Mevalonic acid $40 \pm 6\%$ (p <0.05 when compared with ATV). The co-administration of Mevalonic acid abrogated the cardioprotective effect of Atorvastatin.

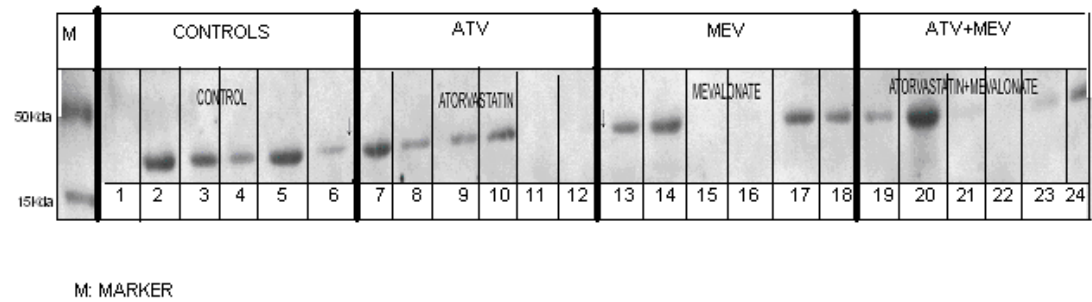
7.3.2 REPERFUSION SALVAGE: WESTERN BLOTS

Blots were performed for which 24 Spargue-Dawley rats were used. 3 hearts were not included as there was a problem with the Langendorff apparatus.

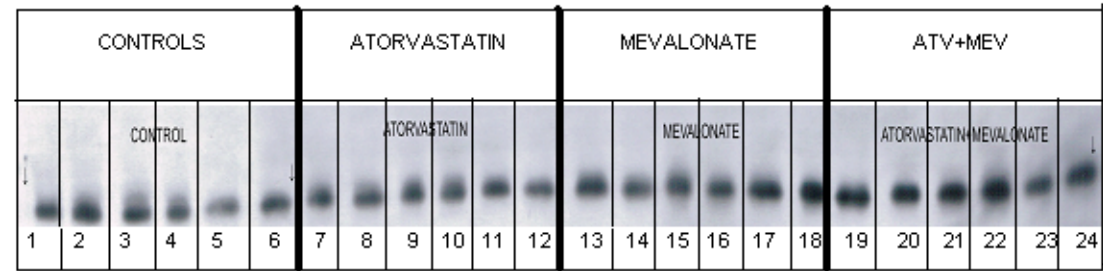
Results were expressed as a ratio of active RhoA to total Rho A and the total Rho A was normalized with β -actin (**Figure 7.03 and 7.04**).

Western Blot Scans:

(a) Active RhoA Blots (After processing for Active Rho-A):



(b) Total RhoA Blots:



(c) Beta-actin Blots:

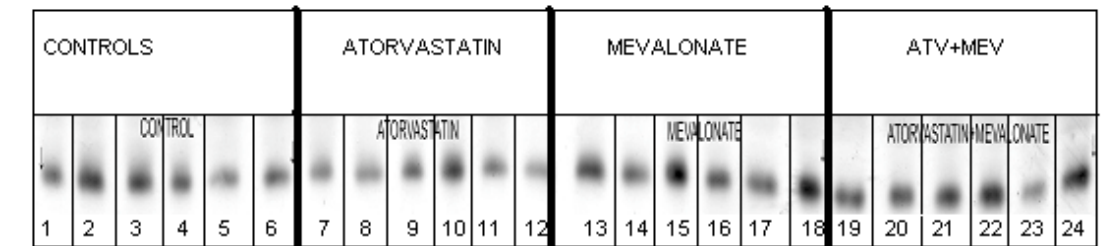


Figure 7.03: Scanned Images of Western Blots.

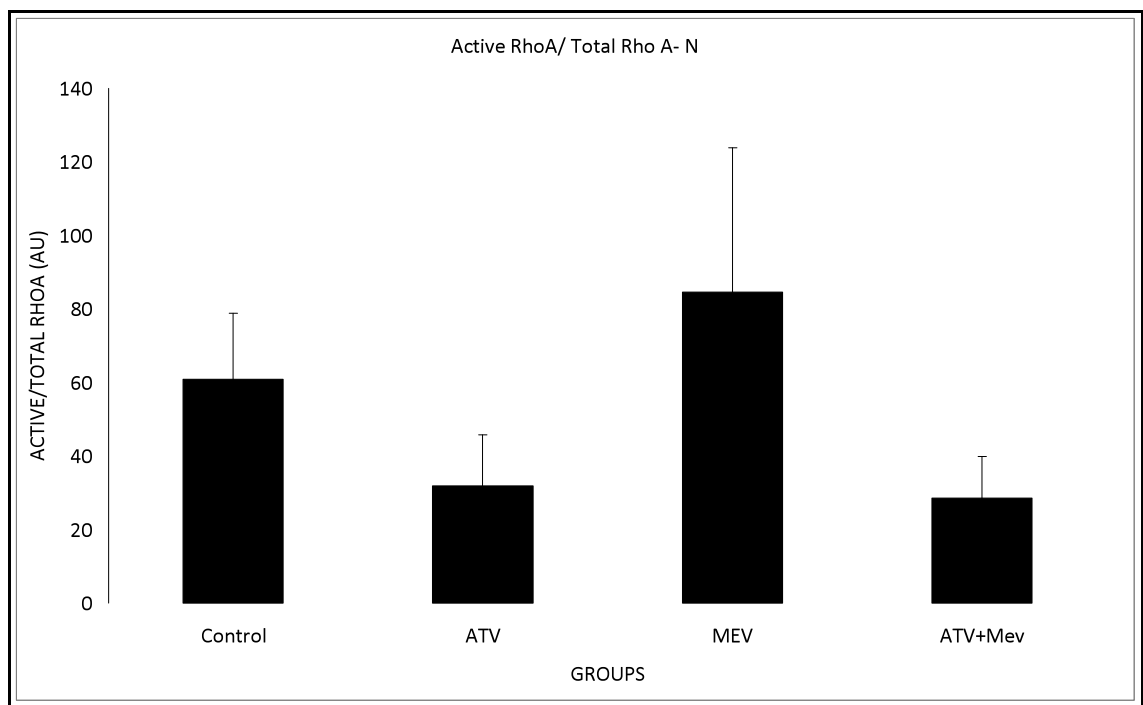
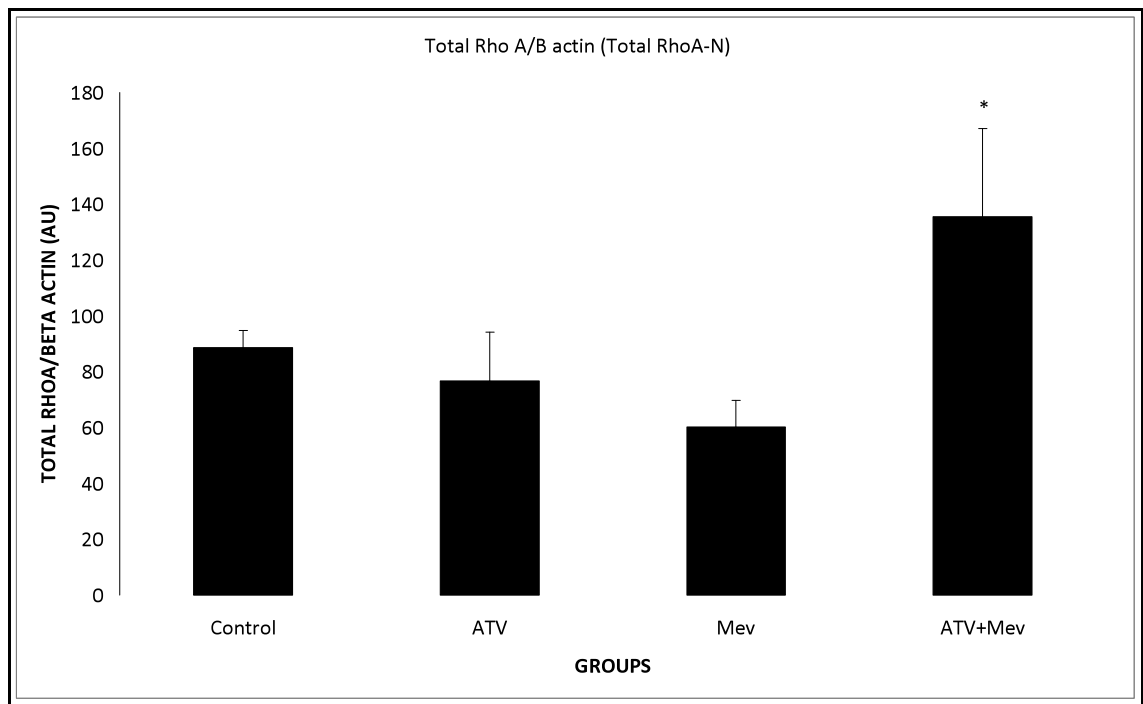


Fig 7.04: Western blot results for Rho A. Total RhoA-N: Total rhoA-normalized. * p value <0.05 when compared to ATV and <0.01 when compared to MEV.

These results demonstrate that Atorvastatin treated hearts have a reduced level of active rhoA and mevalonic acid treated hearts have a higher level of active rhoA but hearts treated with the combination of Atorvastatin and mevalonic acid

did not show a higher level of activated rhoA. However none of these results were statistically significant.

7.4 DISCUSSION

Statins inhibit HMG-CoA reductase, the rate limiting enzyme in the cholesterol biosynthetic pathway²¹³. This enzyme catalyzes the conversion of HMG-CoA to mevalonic acid³⁶⁰. Mevalonic acid is the common precursor to both cholesterol and the isoprenoids. These isoprenoids include farnesylpyrophosphate and geranylgeranyl pyrophosphate²¹³. These serve as important lipid attachments for the post translational modification of various proteins including Rho³⁶¹. Small G proteins of the Rho family comprises 20 members in mammals³⁶². Rho proteins regulate many cellular processes such as cytoskeletal and cell adhesion, cell polarity, endocytosis, vesicle trafficking, progression through the cell cycle, cell differentiation, oncogenesis and gene transcription³⁶³. These Rho proteins act as a molecular switch and switch between an active GTP bound form and an inactive GDP bound form³⁶⁴ (**Figure 7.05**).

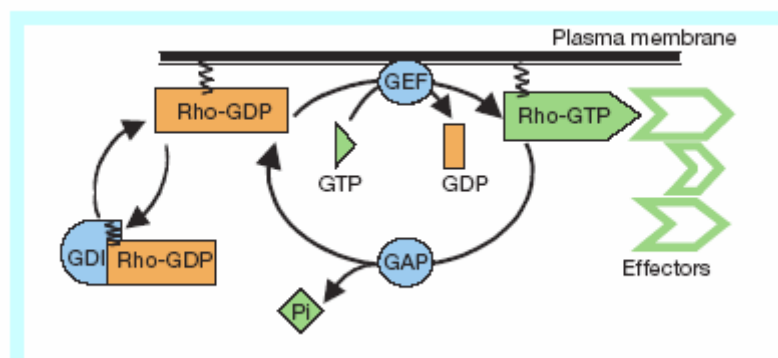


Figure 7.05: The Rho proteins alternate between an active GTP bound state and an inactive GDP bound state. The GEF (Guanine nucleotide exchange factor) mediates nucleotide exchange and thus activation. GAP (GTPase Activation Protein) catalyze hydrolysis of the GTPase and thus inactivation. The GDI (Guanine nucleotide exchange inhibitors) removes the inactive Rho protein from the membrane. Prenylation is essential for the translocation of the protein to the cell membrane. Taken from Etienne-Manneville et al³⁶³.

All Rho proteins are prenylated at their carboxy terminus and this requires the isoprenoid intermediates³⁶⁵. These prenylated proteins then translocate to the cell membrane in the GTP bound state and activate their effectors. The Rho proteins Rho A, Cdc 42 and rac proteins are the best characterized and of these the Rho A protein and its downstream effectors are the focus of cardiovascular protection³⁶⁴. The Rho A protein, in addition to being prenylated and activated by binding to GTP, is also regulated by phosphorylation. Phosphorylation at the Serine 188 site by cAMP/cGMP dependent kinases (PKA and PKG) results in binding to GDI and thus extraction of the protein from membrane and inactivation³⁶⁶.

The effectors of Rho A include the Rho associated coiled-coil Kinase (ROCK), Rhotekin, mDia-1 and PKN²⁰¹. Of these ROCK is the most prominent and most well studied and has a role in myocardial reperfusion injury³⁶⁷. ROCK is a serine–threonine kinase that mediates many of the important downstream functions of Rho A²⁵⁶. ROCK consists of two isoforms: ROCK1 (ROK β) and ROCK2 (ROK α)³⁶⁸. ROCK1 is highly expressed in kidney, liver, spleen, lung and testis while ROCK2 is preferentially expressed in cardiac and brain tissue³⁶⁸.

More than 15 substrates have been identified for ROCK²⁰¹. ROCK targets the myosin phosphatase in vascular smooth muscles and thus results in calcium sensitization and vascular smooth muscle cell contraction³⁶⁹. It also phosphorylates troponin in cardiomyocytes and increases the tension generated during contraction³⁷⁰. Phosphatase and tensin homologue (PTEN) is a target of ROCK³⁷¹. PTEN is a phosphatase that dephosphorylates both proteins and phosphoinositide substrates and has an important role in the regulation of intracellular signalling especially the PI3Kinase/Akt pathway (involved in the regulation of cell growth, cell survival and protein synthesis)²⁰¹.

ROCK-associated phosphorylation of PTEN results in activation of the phosphatase activity of PTEN which subsequently results in the inhibition of the PI3kinase/Akt pathway²⁰¹. Rho has been shown to activate PTEN in leukocytes and human embryonic kidney and the phosphatase action of PTEN has been shown to be dependent on the action of ROCK³⁷¹. **(Figure 7.06)**

Thus it would seem likely that the acute cardioprotection obtained by the administration could be due to its inhibition of RhoA prenylation which in turn would result in inhibition of ROCK and thus inhibition of the phosphatase activity of PTEN. This would eventually lead to activation of PI3K and eNOS. Indeed experiments in a Langendorff rat heart model has demonstrated an upregulation in PTEN phosphatase activity in chronic Atorvastatin treated hearts and this inhibitory action was overcome by an acute pre-ischaemic administration of Atorvastatin in a rat Langendorff model²⁴⁴. This partly formed the basis of my hypothesis.

ROCK has also been shown to be responsible for reduced endothelial Nitric Oxide synthase (eNOS) activity during hypoxia in human endothelial cells by reducing stability of the eNOS mRNA and also reducing the phosphorylation of eNOS²⁵⁴. This would suggest a more direct mechanism by which ROCK could inhibit components of the RISK pathway. ROCK has also been shown to inhibit phosphorylation of PI3kinase in vascular smooth muscle cells³⁷². **(figure 7.07).**

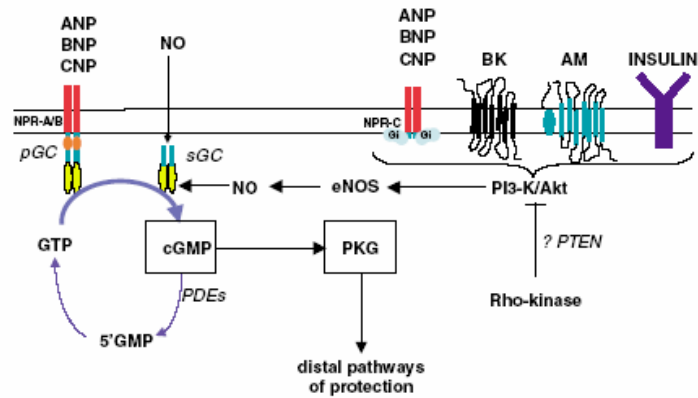


Figure 7.06: ROCK, PTEN and NO: ROCK possibly inhibits the action of PI3 kinase through PTEN. This in turn results in reduced eNOS phosphorylation, a reduction in NO and a subsequent reduction in Protein Kinase G (PKG), which eventually results in activation of the MPTP. Taken from Burley et al³⁷³.

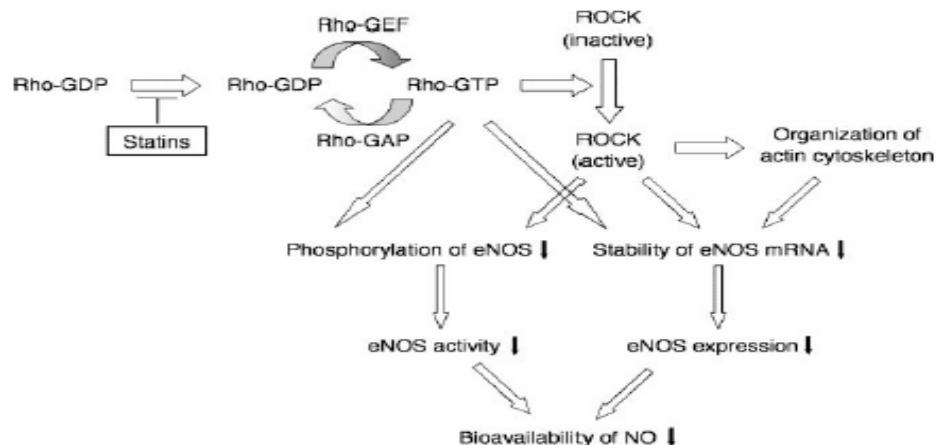


Figure 7.07: ROCK and NO: ROCK results in reduced stability of eNOS mRNA and reduced phosphorylation of eNOS. This is dependent on the activation of Rho A. Taken from Rikitake et al³⁷⁴.

ROCK inhibition with Fasudil has been shown to attenuate infarct size in a rat in-vivo model when administered prior to ischaemia and this is associated with an increase in PI3 kinase and Akt activity²⁵⁶. Fasudil was also shown to reduce infarct size in a Langendorff perfused rat heart model when administered during reperfusion and this was due to an Akt/eNOS dependent mechanism²⁰³. There is also evidence in diabetic in vivo and in vitro rat heart studies to suggest that acute inhibition of RhoA and ROCK is associated with increase contractile

activity³⁷⁵. Thus there is good evidence to suggest that acute inhibition of ROCK during reperfusion is cardioprotective. This would further lend support to the hypothesis that Atorvastatin-induced cardioprotection is likely to be due to RhoA/ROCK inhibition.

In a rat in-vivo model, Rosuvastatin administered prior to ischaemia has been shown to reduce infarct size and this effect was abolished by the addition of geranylgeranyl pyrophosphate (GGPP) which overcomes the statin induced inhibition of rho A, and was also dependent on an increase in the cytosolic component of rho A²⁵⁷. It would thus appear that statin induced inhibition of rho A prenylation is central to its cardioprotective activity. It may also be central to PI3 Kinase/Akt activation in myocardial ischaemia –reperfusion injury. (figure 7.08)

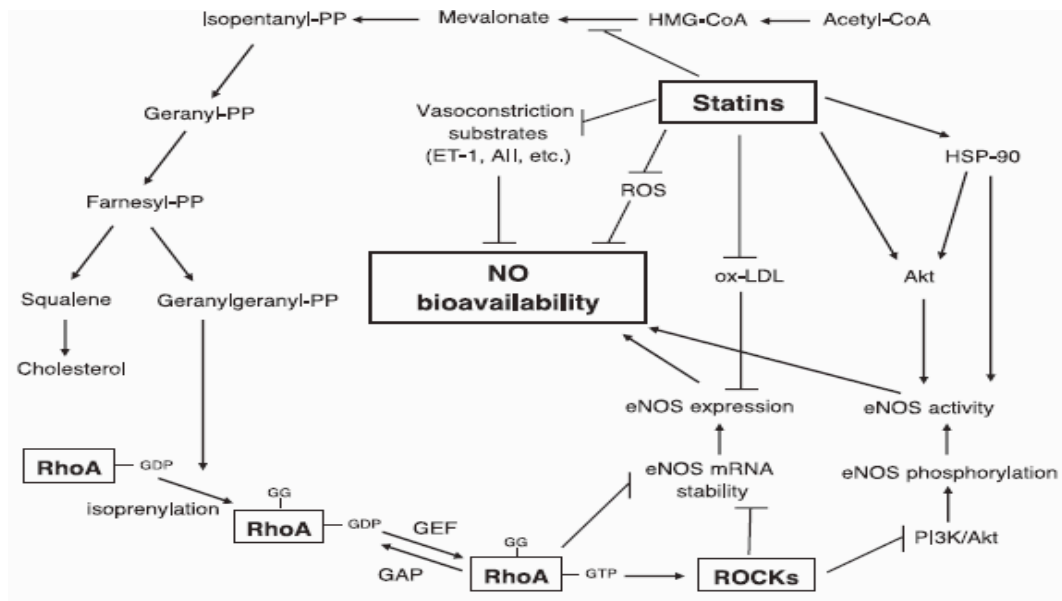


Figure 7.08: Statins inhibit the prenylation of Rho A by a cholesterol independent mechanism. This results in a reduction in Rho A and ROCK which in turn results in activation of PI3Kinase/Akt and eNOS. Taken from Noma et al³⁶⁸.

The experiments described in this section of this thesis demonstrate that the infarct sparing action of Atorvastatin administered during reperfusion is abrogated by the administration of mevalonic acid (the common intermediate for both cholesterol and isoprenoids). However while the Western blots demonstrate a reduced level of active RhoA (negative regulator of PI3 kinase and eNOS) in the Atorvastatin treated group and an increase in activity in the mevalonic acid treated group, none of the values are significant. The Western blot results described in this section are statistically not significant possibly due to following two reasons.

(a) The Rhotekin bead assay to measure active Rho A consists of an immunoprecipitation step which involves colourless slurry. During the multiple washing steps too little or too much of the sample may have been pipetted out to result in large standard errors and some false readings.

(b) In addition the protein sample quantification was carried out in cardiac tissue that formed the 'area at risk'. Thus if the tissue had a large amount of infarct there may have been very little viable tissue to give an accurate quantification of protein activation. This is especially true for the hearts treated with a combination of Atorvastatin & mevalonate which would have a higher infarct rate when compared to Atorvastatin treated hearts. This may be lent support by the fact that experiments in rats treated with Rosuvastatin were analysed for RhoA activation in hearts that were not subjected to ischaemia-reperfusion²³⁹. Active RhoA is membrane bound and if tissue necrosis is quite large, active RhoA quantification may be difficult. This is purely conjecture and based on indirect evidence.

7.5 CONCLUSION:

These experiments demonstrate for the first time that Atorvastatin-induced cardioprotection is due to its inhibition of HMG CoA reductase and that there is a suggestion that this maybe related to RhoA inhibition.

8. SUMMARY OF FINDINGS AND FUTURE DIRECTIONS

8.1 SUMMARY OF FINDINGS

The experiments in this thesis have demonstrated the following:

(a) Atorvastatin is cardioprotective when acutely administered during reperfusion, in the in vitro rat heart model. This is the first time that this has been demonstrated in this model. As mentioned before the isolated rat heart model is a highly reproducible model that can be used to study biological, morphological and pharmacological indices in the absence of neuro-hormonal factors that are present in an in vivo model²⁷⁵. However the disadvantage of this model is that the extrapolation to intact human body may not always be relevant²⁷⁵.

(b) The co-administration of RU486, the GCR blocker, to Atorvastatin treated hearts did not result in a loss of cardioprotection. However the GCR blocker in itself was cardioprotective and thus we were unable to conclude as to whether Atorvastatin acts via the glucocorticoid receptor.

(c) Atorvastatin is cardioprotective when administered to rat heart (in vitro model) prior to ischaemia and at the onset of reperfusion. The protective effect of pre-ischaemic administration of Atorvastatin was abrogated by the non-selective adenosine receptor antagonist, SPT, while co-administration at the onset of reperfusion did not have any effect. It could thus be concluded that Atorvastatin administered prior to ischaemia involves an adenosine dependent cardioprotective mechanism while when administered during reperfusion, it has an adenosine independent mechanism.

(d) Atorvastatin induced cardioprotection is abrogated by the administration of mevalonic acid. Western blot studies for RhoA activation revealed a reduction in active RhoA levels in Atorvastatin-treated hearts but this was not statistically significant. We have thus demonstrated for the first time that Atorvastatin induced cardioprotection in the in vitro rat heart model is due to its inhibition of HMG CoA reductase and that this maybe related to the inhibition of RhoA activation.

8.2 CLINICAL APPLICATION

Statin therapy is currently a part of standard therapy for primary and secondary prevention of Coronary Artery Disease. However it is largely used for its cholesterol beneficial effects and not necessarily at the higher dose necessary for its pleiotropic actions. The insights obtained from the work presented in this thesis will add to the scientific basis for the need to treat patients with acute coronary syndromes with appropriate doses of statins in order to be able to obtain the full infarct sparing effect of statins.

This insight into mechanistic actions will also lead to the discovery of novel therapeutic agents that target the isoprenoid synthetic pathway and thus be used as an effective adjunct to conventional therapy in the setting of acute coronary syndromes. This will also rationalize the therapeutic approach in patients intolerant to higher doses of statins by either co-administration of rho kinase inhibitors or by offering a 'one-off' high dose statin regime during an acute coronary syndrome.

8.3 FUTURE DIRECTIONS

Possible future research could be in the following spheres:

(a) Laboratory research:

- The role of glucocorticoid receptor could be explored further by using mice knock-out models which lack the glucocorticoid response elements for the receptor.
- The role of adenosine in the pre-ischaemic setting could also be explored by using specific adenosine receptor inhibitors and also the use of adenosine transport inhibitors. PKC activation and A_{2B} sensitivity could be specifically explored in order to provide further insight into the mechanistic actions.
- To demonstrate pleiotropy, cholesterol assays could be performed on the coronary effluent and also in the myocardium. If statin-induced cardioprotection were independent of cholesterol metabolism, the cholesterol levels in coronary effluent should essentially remain unchanged. RhoA activity assay could be carried out by alternative methods.
- The experiments described here could be carried out in human atrial trabeculae model to demonstrate that these mechanisms may be relevant in humans. In this model, human atrial trabeculae harvested from patients undergoing CABG are suspended in buffer perfused organ baths and are paced. Contractile activity is measured during simulated ischaemia and reperfusion and recovery of function is calculated. Ischaemia-reperfusion is simulated by alternating between hypoxic buffer and normoxic buffer.

Statins could be acutely perfused on the rig or given just prior to surgery to assess its effect on contractile activity. Better functional recovery would translate into cardioprotection³⁷⁶.

(b) Clinical Research:

- Clinical trials and proof of concept trials could be carried out in patients to assess the benefit of high dose statins in the setting of acute coronary syndromes, CABG and PCI. For this purpose troponin T or I could be used as a surrogate marker of myocardial injury or alternately 'perfusion imaging' using cardiac MRI could be used.
- In CABG patients atrial trabeculae may be used to analyse the pattern of RhoA activation.

COMMUNICATIONS:

(a) Poster presentation in the International Society for Heart Research, European Congress, Athens 2008.

(b) Poster presentation at University College London, Cardiovascular day.

(c) Editorial Comment in Cardiovascular Drugs and therapeutics- The Power of Drug co-administration, smaller doses better outcomes.

Poster:

INTRODUCTION

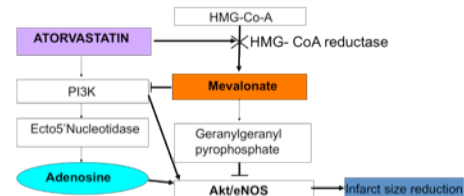
- Reperfusion injury following myocardial ischaemia has been shown to be limited by the use of 3-Hydroxy 3-Methylglutaryl (HMG) co enzyme A (CoA) reductase inhibitors such as atorvastatin (ATV). [1]
- The rapid recruitment of the pro survival kinases such as AKT, following ATV administration, would suggest a non transcriptional action of ATV.
- Atorvastatin may be cardioprotective either by interacting with a receptor or due to its pleiotropic action.
- It has been shown that ATV administered prior to ischaemia, activated ecto5'nucleotidase which increases the production of adenosine, a well established preconditioning agent [2]. Thus ATV administered during reperfusion may also increase the levels of adenosine or directly interact with the adenosine receptor.
- It has been often stated that the cardioprotective action of ATV may be due its pleiotropic action, whereby it reduces the levels of mevalonic acid. Mevalonic acid is a precursor in the cholesterol and isoprenoid biosynthetic pathway and is proposed to be an inhibitor of the pro survival kinases PI3Kinase/AKT.[3]

HYPOTHESES

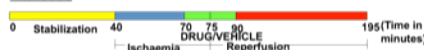
- (1) Atorvastatin, administered during reperfusion, is cardioprotective by either direct interaction with the adenosine receptor or by enhancing the levels of adenosine.
- (2) Atorvastatin, administered during reperfusion, is cardioprotective due to its pleiotropic action whereby it reduces the level of mevalonic acid, a proposed inhibitor of the pro survival kinases.

METHODS & RESULTS

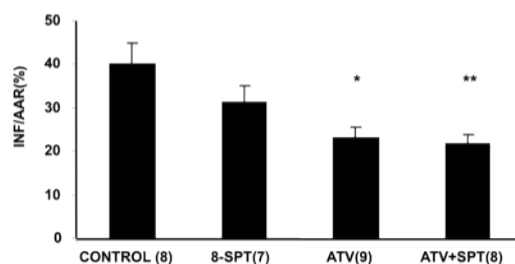
To investigate our two hypotheses, we used Langendorff perfused isolated rat hearts which were subjected to 35 minutes of ischaemia and 120 minutes of reperfusion. Commencing 5 minutes before the onset of reperfusion, the following agents were administered for a total duration of 20 minutes: (a) Vehicle (Control) (b) ATV (c) 8-Sulphophenyl theophylline (SPT, non-specific adenosine receptor blocker) (d) ATV+SPT (e) mevalonic acid (MEV)(f) ATV+MEV. The hearts were injected with Evans blue at the end of the protocol to delineate the non-risk area and then were frozen. These were subsequently sliced from apex to base and then stained by incubating in 10% TTC (triphenyl tetrazolium chloride) and infarct size analysis was carried out using image-J software. Data was represented as a % ratio of the infarct size (INF) to area at risk (AAR). Tests of significance were carried out using the student-t test.



PROTOCOL

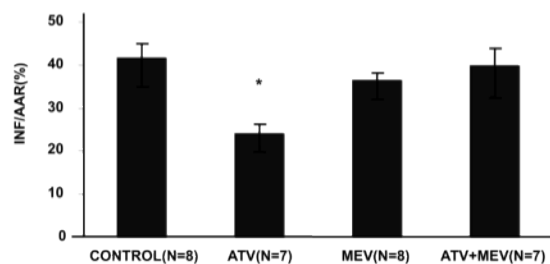


Infarct Size in Male Sprag Dawley myocardium perfused with 8-SPT and atorvastatin



Administration of the adenosine inhibitor 8-SPT along with atorvastatin during reperfusion does not result in inhibition of the infarct size limitation of atorvastatin in the Langendorff system.

Infarct size in Male Sprag Dawley myocardium perfused with atorvastatin and mevalonic acid



Administration of mevalonic acid along with atorvastatin during reperfusion results in loss of the infarct size limitation obtained with atorvastatin. This would suggest that atorvastatin possibly has a pleiotropic action which is responsible for the activation of the prosurvival kinases.

CONCLUSIONS

- We have thus demonstrated that in the in vitro Langendorff system, the cardioprotection afforded by atorvastatin when given acutely during reperfusion is not lost when the non specific adenosine receptor blocker is used. This indicates that the cardioprotective action of atorvastatin during reperfusion may not involve adenosine. This may represent the existence of two distinct mechanisms in two different settings i.e. atorvastatin administered prior to ischaemia and atorvastatin administered during reperfusion.
- We have also demonstrated for the first time that the cardioprotection of atorvastatin when administered during reperfusion is possibly due to its pleiotropic action. However western blot assays are required to further dissect the potential downstream targets of this pleiotropic action.

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